# Suppressing mutant p53 using RNA interference: Investigating a novel potential gene therapy strategy for Colorectal Cancer

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Medicine by

**Caroline Ruth Prior**

April 2012

***Dedication***

To my beloved Nanna,

Doreen Scott (1926 – 2012)

For teaching me that nothing is more important than family and for always believing that my goals were achievable.

*Abstract*

**Suppressing mutant p53 using RNA interference: Investigating a novel potential gene therapy strategy for Colorectal Cancer**

Colorectal cancer is a common disease that kills half of those affected within five years of diagnosis. A pathway of successive genetic mutations leads to tumour progression and p53 has an important and frequent role to play in the late stage of this carcinogenesis. Single nucleotide p53 mutations may lead to a gain of malignant function which may render colorectal cancers less responsive to adjuvant chemotherapy regimes. The technology of RNA interference (RNAi) allows targeted gene silencing and vectors are available that allow stable and prolonged suppression of the protein product under investigation.

I hypothesised that inhibiting mutant p53 in colorectal cancer cells may reduce malignant potential and augment response to adjuvant therapies. The aim was to utilise RNA interference via the pSUPER (oligoengine) plasmid to achieve stable down regulation of mutant p53 protein levels in a panel colorectal cancer cell lines in culture and to consequently study the effects of this on cell proliferation and response to chemotherapeutic agents.

In total over one hundred clones were produced and analysed, with clonal populations created in three separate cell lines, each of which showed successful down regulation of the mutant p53 protein.

The effect of mutant p53 suppression on the rate of cell proliferation in the SW620 cell line was studied. Whilst some of the clonal populations with down regulation of mutant p53 expression did show a markedly reduced proliferation rate, this was not consistent across all suppressed clones. Some with no alteration in mutant p53 levels also exhibited a degree of slowed proliferation.

Two SW620 clones with significant suppression of their mutant p53 protein levels demonstrated an $IC_{50}$ value half that of the parent population when treated with oxaliplatin. Thus, half the amount of this chemotherapeutic agent was needed to produce the same effect when the mutant p53 protein had been suppressed. The results with the second agent, 5- fluorouracil, were less consistent. Whilst there was a general trend towards lower $IC_{50}$ values in the down regulated clones, this was not always the case.

The overall conclusion from our work is that suppression of mutant p53 may improve the chemotherapy response of colorectal cancer cells, especially to oxaliplatin. However, these experiments show that extrapolating beyond the results obtained for an individual clone can be misleading and that large numbers of clone replicates must be studied before appropriate conclusions can be drawn.

**Acknowledgements**

I would like begin by thanking my primary supervisor Dr. Mark T. Boyd, for initially giving me the opportunity to join his research group, for freely sharing his scientific knowledge and providing guidance and support throughout the preparation of this thesis. I would also like to thank my clinical supervisor Mr. Paul Rooney, for encouraging me to study for an MD in the first place and for mentoring me both in the oral presentation of my thesis findings and more generally in my clinical career progression. I thank Professor Neoptolemos and Alison Waghorn for facilitating the opportunity to undertake this research alongside my surgical career. I thank my 'lab. buddies' Dr. Maria Maguire and Dr. Mark Brady for turning me from a disorganised (and messy!) beginner into a 'not bad for a medic' scientist. I must formally thank Professor Joe Milner from the York Cancer Research laboratory for the HCT116 cells and their derivatives as well as Royal Liverpool University Hospital Research and Development Fund for generously supporting this project. I thank Dr. Bill Greenhalf and Dr. Carlos Rubbi for their help with data interpretation and the dreaded statistics! More generally I would like to show my appreciation to all the members of the Boyd laboratory who helped along the way and to the other members of the surgical division who provided clerical and administrative support (especially those who were in the lunchtime crossword club!). Finally the people who have really borne the brunt of the late nights, foul moods etc.....

Mam and Dad, for all the practical support with child-care, proof reading etc. but more importantly for always knowing it would one day get finished and providing unconditional love in the mean time – my heartfelt thanks.

To my husband Chris, what can I say; there is no way I would be sitting here writing this, the last page of my thesis to be completed, if it wasn't for you. You are and always have been my rock and what you sacrifice for me to accomplish my goals always astounds me, but never goes unnoticed. I guess that's what soul mates are for?

Finally my two beautiful children Emily and Daniel; you were both twinkles in our eyes when my MD studies began. Writing up with two little ones has not always been plain sailing but the laughter and fulfilment you have brought to my life is worth all the three AM finishes in the world – mummy loves you very much!

## *List of Tables*

## *List of Figures*

## *List of Abbreviations*

| | |
|---|---|
| 5FU | 5 fluorouracil |
| AJCC | American Joint Commission on Cancer |
| APC | Adenomatous Polyposis Coli |
| APS | ammonium persulphate |
| BME | betamecapnoethanol |
| BMI | Body Mass Index |
| CDKs | cyclin dependent kinases |
| CI | confidence interval |
| CIN | classic chromosomal instability |
| CRC | colorectal cancer |
| DBD | DNA-binding domain |
| DMSO | dimethyl sulphoxide |
| dsRNA | double stranded RNA |
| ECL | enhanced chemo-luminescence |
| EDTA | ethylenediaminetetraacetic acid |
| eGFP | Enhanced Green Fluorescence Protein |
| FAP | Familial Adenomatous Polyposis |
| FBS | foetal bovine serum |
| FITC | fluorescein isothiocyanate |
| FOBt | Faecal occult blood test |
| HEPES | N-2-hydroxyethylpiperazine-N'-ethane sulphonic acid |
| HIV | Human immunodeficiency virus |
| HNPCC | Hereditary Non-Polyposis Colon Cancer |
| HRP | horse radish peroxidase |
| HRT | Hormone Relacement Therapy |
| JPS | juvenile polyposis |
| LOH | loss of heterozygosity |
| mAb | monoclonal antibody |
| MAC | Modified Astler-Coller |
| MMR | DNA mismatch repair |
| MSI | micro-satellite instability |
| MSI-H | high-frequency microsatellite instability |

| | |
|---|---|
| MSS | microsatellite stability |
| MAP | MUTYH-associated polyposis |
| NICE | National Institute for Clinical Excellence |
| NSAIDs | non-steroidal anti-inflammatory drugs |
| pAb | polyclonal antibody |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PGK | phosphoglycerate kinase |
| PJS | Peutz-Jeghers syndrome |
| PMSF | phenylmethanesulfonyl fluoride |
| RISC | RNA Induced Silencing Complex |
| ROS | reactive oxygen species |
| RR | relative risk |
| RLU | Relative Light Units |
| RNA | ribose nucleic acid |
| SDS | sodium dodecyl sulphate |
| SEM | Standard error of the mean |
| shRNA | short hairpin RNA |
| siRNA | small interefering RNA |
| SLIP | Stuart Linn immuno-precipiation |
| ssRNA | single stranded RNA |
| TEMED | N,N,N',N' – tetramethylethylenediamine |
| TNM | Tumour, Node, Metastasis |
| X-gal | 5-bromo-3-indoyl-β-D-galactopyranoside |
| β-gal | β-galactosidase |

# Chapter 1. Introduction

Colorectal cancer is a common disease that still kills half of those affected within five years of diagnosis. A pathway of successive genetic mutations leads to tumour progression and p53 has an important and frequent role to play in the late stage of this carcinogenesis. Single nucleotide p53 mutations may lead to a gain of malignant function above and beyond that witnessed with loss of the wild-type p53 phenotype. This may render colorectal cancers less responsive to adjuvant chemotherapy regimes. The technology of RNA interference allows targeted gene silencing and vectors are available that allow stable and prolonged suppression of the protein product under investigation. This thesis will examine whether these vectors can be successfully used on colon cancer cell lines in vitro to suppress mutant p53 and hence abrogate any 'gain of function' effects. It will then consider whether such suppression effects cell proliferation and improves responsiveness to chemotherapeutic agents within these cell lines.

This introduction considers colorectal cancer as a clinical disease; detailing its epidemiology, aetiology, natural history and staging. The current methods for screening, diagnosing and treating the disease are reviewed and survival statistics are considered. The mechanism of action of chemotherapy is detailed along with consideration of some the agents used in colorectal cancer treatment and the current role chemotherapy plays in the neo-adjuvant, adjuvant and palliative settings.

The genes and pathways implicated in inherited and sporadic colorectal cancer tumorigenicity are reviewed with establishment of the role p53 plays within this network. The study then focuses in on the *TP53* gene itself, reviewing some of the

extensive research of the past thirty years into the gene's structure and function. The implications of *TP53* mutations in colorectal cancer and the effects on prognosis and treatment will be discussed. The nature and relevance of 'gain of function' p53 mutations will be highlighted with a review of the published work on this topic to date. The technology of RNA interference and its role in the laboratory and clinical settings to date will also be considered.

This section will conclude with the hypothesis and the aims for this MD thesis being presented.

## 1.1 Colon cancer as a disease

### 1.1.1 Epidemiology

Colorectal cancer is a major cause of mortality and morbidity in the Developed world. Worldwide an estimated 1.24 million new cases of colorectal cancer were diagnosed in 2008, accounting for more than 9% of all new cancer cases [1]. In 2008 there were 39,991 new cases of large bowel cancer registered in the UK, resulting in around 110 new cases of colorectal cancer being diagnosed each day. Around two-thirds of these (25,551) arise in the colon and one-third (14,440) in the rectum. In 2010, there were 16,013 deaths from bowel cancer in the UK [2-5]. The lifetime risk for men of being diagnosed with colorectal cancer in the UK is estimated to be 1 in 15 and for women 1 in 19. In the UK colorectal cancer is now the third most common cancer in women after breast and lung cancer whereas in men it also ranks third after prostate and lung cancer [6-8].

The occurrence of colorectal cancer is strongly related to age, with 86% of cases arising in people who are 60 years or older. The data shown in Figure 1.1 indicates that until age 50, men and women have similar rates for colon cancer, but in later life male rates are higher. In numerical terms, there are more male cases of bowel cancer in almost all age-groups up to the age of 84, after which female cases are in the majority, even though their rates are lower, as women make up a larger proportion of the elderly population. Overall the male to female ratio is 11:10. The incidence of rectal cancer is higher in men whilst the incidence of colon cancer is higher in women [2-5].

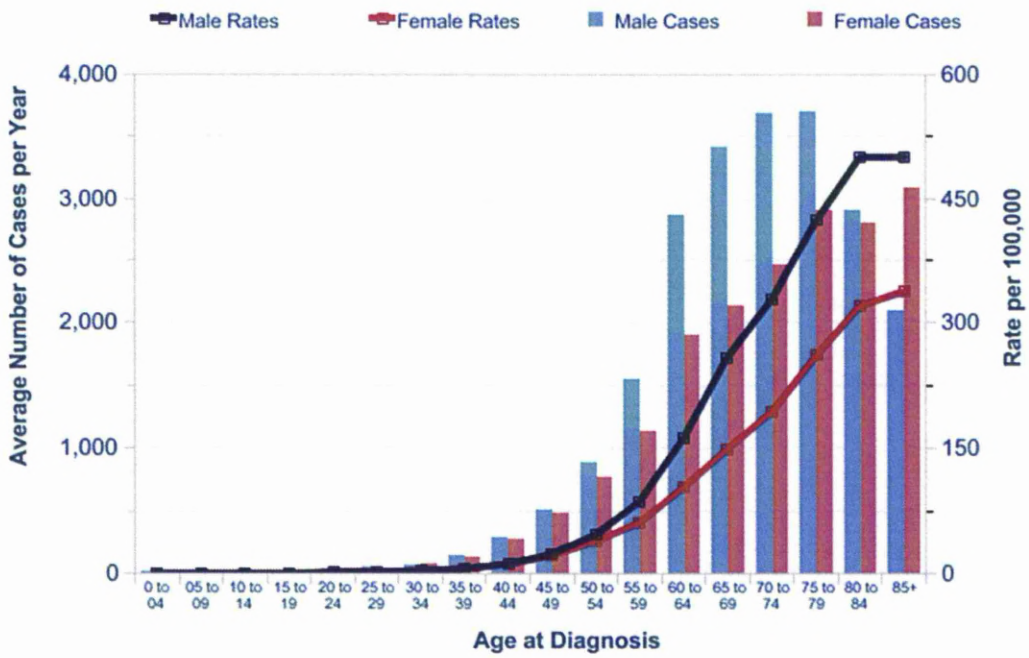



***Figure 1.1: Colorectal Cancer: Average Number of New Cases Per Year and Age-Specific Incidence Rates, UK , 2006-2008.***

***Published by Cancer Research UK.***
***http://info.cancerresearchuk.org/cancerstats/types/bowel/***

The lifetime risk of a man in Great Britain developing colorectal cancer has almost doubled between 1975 and 2008, from 3.5% (or 1 in 29 men) to 6.9% (or 1 in 15).

In women the risk has increased from 3.9% (or 1 in 26 women) to 5.4% (or 1 in 19 women).

Incidence rates vary across the country, particularly in men. Incidence rates are as low as 50 per 100,000 men in London, which along with the South East and the East of England, has rates below the England average. However, in the North East the incidence rate is more than 65 per 100,000 men and along with the North West and the West Midlands, it has rates significantly higher than the England average. Female incidence rates across the country are much closer to the England average with London being the only region which is significantly lower at 35 per 100,000 women [9].

There is a deprivation gradient for male colorectal cancer patients, with incidence rates 11% higher in the most deprived groups than in the affluent groups [10].

Bowel cancer is the third most common cancer worldwide after lung and breast with almost 60% of all colorectal cancers occurring in the more developed regions. Globally there are large variations in rates, with the lowest rates in Africa, Asia and Latin America and the highest in Western Europe, Northern America and Australasia[1]. Japan is an exception to other countries in the Eastern Asia region with age-standardised rates similar to other highly developed countries. Countries that have had a rapid 'westernisation' of diet, such as Japan, have also seen a rapid increase in the incidence of colorectal cancer. Consumption of meat and dairy products in Japan increased ten-fold between the 1950s and 1990s [11, 12]. Epidemiological studies report a rapid increase in risk for colorectal cancer in migrants moving from low to high risk countries [13] and the rates for second

generation migrants can be double that of first [14]. The risk of colorectal cancer also decreases after migrating from a high to a lower risk country. The magnitude of incidence variation worldwide, together with migration data, suggest that many cases of colorectal cancer could be prevented.

In Europe, the incidence of colorectal cancer is increasing, particularly in southern and eastern Europe, where rates were originally lower than in western Europe [15, 16]. In the USA, the incidence rose until the mid-1980s but in the last two decades rates have fallen for both men and women [17].

Although patients diagnosed with early stage disease have a high cure rate, many patients present later when long term survival is poor. The five-year relative survival rates for both male and female colon and rectal cancer have doubled between the early 1970s and the mid 2000s. In the early 1970s, five-year relative survival for male colon cancer was 22% (23% for women) and this rose to 50% (51% for women) in the mid 2000s. Over the same time period, the five-year survival rates for male rectal cancer rose from 25% to 51% and from 27% to 55% for female rectal cancer [18]. Ten-year survival rates are only a little lower than those at five-years indicating that most patients who survive for five years are cured from this disease.

### 1.1.2 Aetiology

#### *1.1.2.1 Genetics*

The development of colorectal cancer results from the interactions of an individual's genotype and the environmental influences which they encounter during their lives. As it is a common cancer, many people are quite likely by chance alone to have at least one affected relative [19], as the number of affected relatives increases, so does the risk of the individual developing the disease [20]. There is a spectrum of genetic

risk from those with no particular genetic disposition through to those who will almost inevitably develop colorectal cancer. There are various degrees between these extremes where the contribution of inherited genetic risk plays a variable role. In order to stratify these risks into categories for screening strategies, the genetic inheritance related to colorectal cancer is considered as high, high-moderate or low-moderate risk.

High risk patients, 3 - 5% of the total cases [21], are those in whom a family member is known to have one of the 'inherited bowel cancer syndromes" i.e. Familial Adenomatous Polyposis (FAP), MUTYH-associated polyposis (MAP), juvenile polyposis (JPS), Peutz-Jeghers syndrome (PJS) or Hereditary Non-Polyposis Colon Cancer (HNPCC) aka Lynch Syndrome. These high penetrance genetic disorders are identified on the basis of one or more of the following criteria:

- A family history consistent with an autosomal dominant cancer syndrome.
- Pathognomonic features of a characterised polpyposis syndrome personally or in a close relative.
- The presence of a germline pathogenic mutation in a colorectal susceptibility gene.
- Molecular features of a familial syndrome in a colorectal cancer arising in a first-degree relative.

The lifetime cancer risk for theses syndromes ranges from 10-100% and so the intensity of surveillance reflects that elevated level of risk and the natural history of the resultant neoplasia [22].

In FAP family members, annual flexible sigmoidoscopy and alternating colonoscopy should be offered to mutation carriers from diagnosis until polyp load indicates a need for surgery. Patients should be advised to undergo prophylactic surgery (ideally panproctocoloectomy) between the ages of 16 and 25 years, but the exact timing of surgery should be guided by polyp numbers, size and dysplasia alongside consideration of educational and child-bearing issues [23].

In Lynch syndrome (HNPCC) family members, total colonic surveillance, at least biennially, should commence at age 25 years and continue to age 70-75 years, or until clinical co-morbidity makes it clinically inappropriate. Colonoscopic surveillance for individuals with proven MMR gene mutations has been shown in retrospective case-control comparisons to provide an average of seven years of extra life [24].

The high-moderate risk category comprises:

- People with three or more affected relatives in a first-degree kinship with each other (none less than 50 years old, otherwise they would fulfil high risk criteria) or
- Two affected relatives less than 60 years, or with a mean age less than 60, in first degree kinship with each other.

People in the high-moderate risk category merit low intensity surveillance comprising 5-yearly colonoscopy commencing at age 50 until age 75years.

The low-moderate risk group comprises those with only one affected relative less than 50 years old or only two affected first degree relatives aged 60 or older. These individuals are recommended for a once-only colonoscopy at age 55; with further

surveillance undertaken only if adenomatous polyps are encountered at this investigation [25].

The low risk group encompasses all those in whom the above family history criteria are not satisfied. Although the risk of developing the disease may be up to twice that of the average population, there is no evidence to support routine invasive surveillance in this group. The risks of colonoscopy as a procedure are not offset by the detection rate of new cancers in asymptomatic patients [26].

#### *1.1.2.2 Lifestyle Factors*

A high intake of dietary fibre, in particular cereal fibre and whole grains, is associated with a reduced risk of colorectal cancer [25]. It is hypothesised that reduced intestinal transit time and the effects of dilution could reduce the exposure of the large intestine mucosa to potential pathogens [27]. The effects of wheat fibre on mucosal cell turnover, [28] epidemiological [29] and dietary questionnaire studies [30] all back up this hypothesis. The effects of red meat have been examined in many epidemiologic studies; most, though not all, associate an increase in colon cancer or adenoma risk with greater intake of red meat. In the European Prospective Investigation into Cancer and nutrition (EPIC) study; the absolute risk of developing colorectal cancer within 10 years for a 50 year old participant was 1.71% for the highest category of red and processed meat intake compared with 1.28% for the lowest category of intake [31]. It is suggested that animal fats cause an increased excretion of bile salts in the faeces but also promote the growth of bacteria that can degrade bile salts into carcinogens [32]. Secondary bile acid secretion is increased after cholecystectomy and this was said to correlate with a rise in colorectal cancer incidence [33]. However, a more recent study refutes this finding [34].

Calcium binds bile acids and two large scale, double-blind, randomised controlled trials have shown that dietary calcium supplementation can bring about a moderate reduction in the risk of recurrent colonic adenomas. However a Cochrane review concluded there was insufficient evidence to recommend the general use of calcium supplements to prevent colorectal cancer [35]. Diets lacking in vegetables have been shown to increase risk [36]. Isiocyanates in cruciferous vegetables (e.g. broccoli) may help induce apoptosis in neoplastic cells [37, 38]. High glycaemic dietary load and high intake of refined carbohydrates have been shown to increase risk, potentially secondary to insulin resistance [39, 40].

There is a consistent inverse relationship between exercise and colon cancer risk in all studies, though the effects are greater in men than women [41]. Elevated BMI also seems to independently increase risk of both adenomas and colorectal cancer [42]. A recent meta-analysis has confirmed the link between smoking and colorectal cancer, with relative risk increased proportionate to daily consumption, age at initiation, duration and total number of pack years [42]. The relationship between alcohol and cancer has been controversial, but most evidence indicates that high intake of alcohol increases risk of colorectal cancer. A pooled analysis of 8 prospective cohort studies, which included nearly 500,000 participants, yielded a multivariate risk of colorectal cancer of 1.24 (95% CI 1.07–1.42) for consumption of ≥30 g/day of alcohol compared to low intake [43]. There is substantial evidence that the use of non-steroidal anti-inflammatory drugs (NSAIDs) and aspirin are associated with a reduced risk of colorectal cancer and adenomatous polyps [44, 45].

Use of Hormone Replacement Therapy (HRT) in post-menopausal women has been associated with a reduced risk of developing colorectal cancer. In the Women's Health Initiative trial (16,608 participants); relatively short-term use of oestrogen plus progestin was associated with a decreased risk of colorectal cancer [46]. There were 43 invasive colorectal cancers in the hormone group and 72 in the placebo group (hazard ratio, 0.56; 95 percent confidence interval, 0.38 to 0.81; P=0.003). However, the increased risk for other types of cancer and cardiovascular effects indicate that HRT cannot safely be used for chemoprevention [47].

### 1.1.3 Natural History / Pathology

In normal colon tissue, stem cells at the bottom of crypts divide continuously in an asymmetric fashion, adding cells to the crypt's proliferative area [48]. These intermediary cells will either differentiate further into goblet cells or migrate towards the lumen [49]. In malignant tissue, genomic alterations disrupt normal division, resulting in symmetric division, with the possible result that both daughter cells may preserve the clonogenic traits of the stem cell. If apoptotic activity is not adequate, this leads to the build up of altered stem cells and the risk of clonal expansion [49], see Figure 1.2. Aberrant crypt foci are one of the foremost pre-malignant lesions seen in colorectal cancer. Dysplastic aberrant crypt foci are the precursors of adenomatous polyps. Once developed the small adenoma enlarges and becomes increasingly dysplastic. Low grade dysplastic adenomas exhibit mild to moderate architectural irregularities. High grade dysplastic adenomas show heightened changes in glandular patterns and / or extreme cytological irregularities. High grade dysplasia is the catalyst for malignant transformation [50]. Histologically, adenomas are classified

as tubular, tubulo-villous or villous, with increasing villous content and neoplastic tendency respectively [51].

Although the majority of adenomas diagnosed are polypoid or exophytic, the flat adenoma (defined as an adenoma where the depth of dysplastic tissue is no more than twice that of the mucosa) is now a recognised entity. These lesions may account for up to 40% of adenomas, are premalignant and may have a greater tendency towards malignant transformation than polypoid adenomas [52].

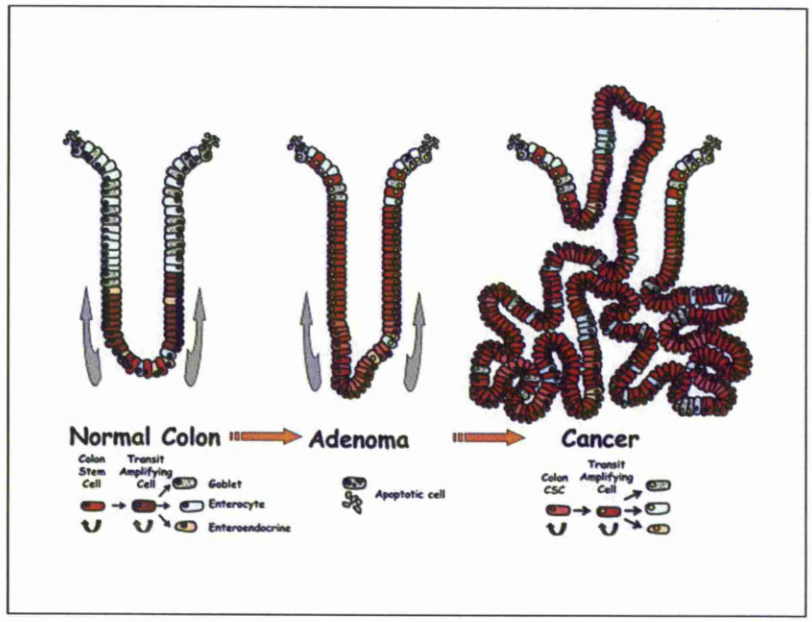



***Figure 1.2:*** *Model for colon cancer initiation from stem cells.*

***Reproduced from Chandler and Lagasse, Stem Cell Research and therapy 2010 [53].***

Accurate, detailed and consistent pathology reporting for colorectal cancer is important for estimating prognosis and planning further treatment in terms of adjuvant therapy. The Dukes' classification was first published in 1932 and is still utilised today [54]. This simple classification based on pathological findings was modified further by Dukes in 1958, sub-dividing group C to detail apical node involvement [55], as shown in Table 1.1. An adaptation by the Americans Astler and

Coller in 1954 further divided stages B and C [56], as shown in Table 1.2. These staging systems are still widely quoted, but the TNM (Tumour, Node, Metastasis) classification, produced by the American Joint Commission on Cancer (AJCC) and currently in its 7th edition, is the internationally preferred method for reporting and staging colorectal cancers [57], which is demonstrated in Table 1.3 and Table 1.4

***Table 1.1: Duke's Original Classification [55]***

| Stage | Description |
|---|---|
| A | Limited to the bowel wall |
| B | Through the bowel wall |
| C | Regional lymph nodes metastasis |
| (later divided into C1 Apical node negative and C2 where the node is positive) | |

***Table 1.2: Modified Astler-Coller (MAC) staging system [56]***

| Stage | Description |
|---|---|
| A | Limited to the mucosa |
| B1 | Tumour extending into but not through the muscularis propria |
| B2 | Tumour penetrating through the serosa but no involvement of lymph nodes |
| B3 | Tumour invades adjacent structures |
| C1 | Same as B1 plus regional nodal metastasis |
| C2 | Same as B2 plus regional nodal metastasis |
| C3 | Same as B3 plus regional nodal metastasis |
| D | Distant metastasis |

*Table 1.3. The TNM Classification of Colorectal Cancer (7th Edition)[57]*

| **Primary tumor (T)** | |
|---|---|
| TX | Primary tumor cannot be assessed |
| T0 | No evidence of primary tumor |
| Tis | Carcinoma in situ: intraepithelial or invasion of lamina propria |
| T1 | Tumor invades submucosa |
| T2 | Tumor invades muscularis propria |
| T3 | Tumor invades through the muscularis propria into the pericolorectal tissues |
| T4a | Tumor penetrates to the surface of the visceral peritoneum |
| T4b | Tumor directly invades or is adherent to other organs or structures |
| **Regional lymph nodes (N)** | |
| NX | Regional lymph nodes cannot be assessed |
| N0 | No regional lymph node metastasis |
| N1 | Metastasis in 1-3 regional lymph nodes |
| N1a | Metastasis in 1 regional lymph node |
| N1b | Metastasis in 2-3 regional lymph nodes |
| N1c | Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis |
| N2 | Metastasis in 4 or more lymph nodes |
| N2a | Metastasis in 4-6 regional lymph nodes |
| N2b | Metastasis in 7 or more regional lymph nodes |
| **Distant metastasis (M)** | |
| M0 | No distant metastasis |
| M1 | Distant metastasis |
| M1a | Metastasis confined to 1 organ or site (eg, liver, lung, ovary, nonregional node) |
| M1b | Metastases in more than 1 organ/site or the peritoneum |

*Table 1.4: Stage of disease according to TNM classification*

| Stage | T | N | M | Dukes | Modified Astler-Coller |
|---|---|---|---|---|---|
| 0 | Tis | N0 | M0 | -- | -- |
| I | T1 | N0 | M0 | A | A |
| | T2 | N0 | M0 | A | B1 |
| IIA | T3 | N0 | M0 | B | B2 |
| IIB | T4a | N0 | M0 | B | B2 |
| IIC | T4b | N0 | M0 | B | B3 |
| IIIA | T1-T2 | N1/N1c | M0 | C | C1 |
| | T1 | N2a | M0 | C | C1 |
| IIIB | T3-T4a | N1/N1c | M0 | C | C2 |
| | T2-T3 | N2a | M0 | C | C1/C2 |
| | T1-T2 | N2b | M0 | C | C1 |
| IIIC | T4a | N2a | M0 | C | C2 |
| | T3-T4a | N2b | M0 | C | C2 |
| | T4b | N1-N2 | M0 | C | C3 |
| IVA | Any T | Any N | M1a | -- | -- |
| IVB | Any T | Any N | M1b | -- | -- |

Another factor that can affect the outlook for survival is the grade of the cancer. Grade is a description of how closely the cancer resembles normal colorectal tissue microscopically. The scale used for grading colorectal cancers goes from G1 (well differentiated) to G4 (poorly differentiated). The grade is often simplified as either "low-grade" (G1 or G2) or "high-grade" (G3 or G4). Low-grade cancers tend to grow

and spread more slowly than high-grade cancers. Most of the time, the outlook is better for low-grade cancers than it is for high-grade cancers of the same stage.

Invasion and spread of colorectal cancer eventually renders it a terminal disease. This may occur directly or via the lymphatic or haematogenous routes. Direct radial spread may involve the small intestine, stomach, pelvic organs or abdominal wall whilst a retroperitoneal extension may invade the ureter, duodenum, or the posterior abdominal wall. The lymphatic spread of colonic cancer progresses usually from the paracolic nodes along the main colonic vessels to the nodes associated with either cephalad or caudal vessels, eventually reaching the para-aortic glands in advanced disease. The most common site for blood-borne spread of colorectal cancer is the liver, presumably arriving by the portal venous system. Up to 37% of patients may have detectable liver metastases at the time of operation, and around 50% of patients will develop overt metastatic disease at some stage [58].

### 1.1.4 Screening

Prior to introduction of the UK bowel cancer screening programme, colorectal cancer often presented either as an emergency (approximately 20% of patients) e.g. obstruction, perforation or bleeding, or as an incidental finding on routine tests performed for another reason e.g. investigating iron deficiency anaemia [59].

However, a meta-analysis of four randomised controlled trials evaluating mass screening using the faecal occult blood test (FOBt) demonstrated a 15% reduction in bowel cancer specific mortality in the trials using biennial screening [60]. Following these demonstrations of mortality reduction, the Department of Health commissioned

a pilot screening programme to assess the feasibility of using biennial FOBt screening as a population screening tool for bowel cancer in the UK.

The initial UK Pilot programme ran from 2000-2002 and three pilot screening rounds for men and women aged 50–69 years were successfully implemented in Coventry and Warwickshire in England and in Tayside, Grampian and Fife in Scotland. These pilots achieved uptake of screening close to the 60% target, and concluded that key parameters of test and programme performance observed in randomised studies of FOBT screening can be repeated in population based pilot programmes. For every 1000 individuals returning at least one test kit during the first phase of the screening pilot in England, there were approximately three positive results, 958 negative results and 39 unclear results after the first test kit. After a possible two further tests for individuals with unclear results, there was an overall total of approximately 16 positive results and 980 negative results per 1000 individuals returning at least one test kit. Approximately 16 out of every 1000 participants returning a test kit were offered a colonoscopy, with uptake of colonoscopy reaching 78%. Cancer was detected in approximately 10% of individuals undergoing colonoscopy, with adenoma being detected in 40% and nothing abnormal being detected in 50% of individuals [61].

Following a second round of pilot studies conducted between 2003 – 2005, [62] the NHS UK Bowel Cancer Screening Programme started to offer biennial screening to those aged 60 – 69 nationally in April 2006, and is now available across England with a target of completing all initial invitations for screening by the end of 2012.

The NHS is also planning to introduce flexible sigmoidoscopy screening for all men and women when they reach the age of 55.This follows a multicentre randomised controlled trial which found that flexible sigmoidoscopy was a safe and practical test which, when offered only once to the appropriate age group conferred a substantial and lasting benefit [63].

### 1.1.5 Presentation and Diagnosis

The left side of the colon is affected more often than the right, with tumours in the sigmoid colon, recto-sigmoid junction and the rectum, accounting for over half of the total cases, as demonstrated in Figure 1.3. In 4-5% of cases there are synchronous lesions at the time of presentation [59].

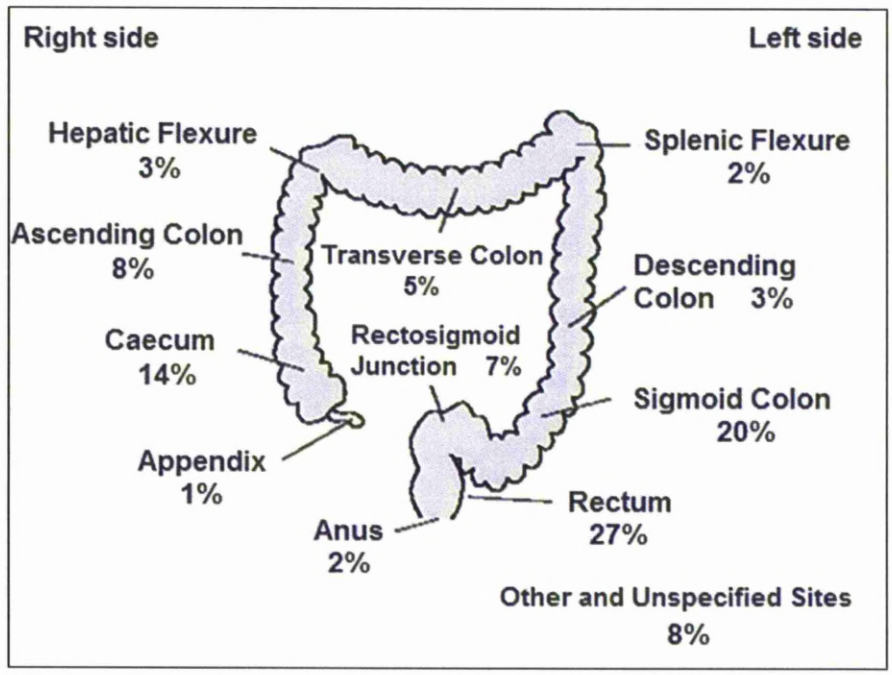



***Figure 1.3: Bowel cancer (C18-21), Percentage Distribution of Cases within the Large Bowel, Great Britain, 2007-2009.***

***Published by Cancer Research UK. info.cancerresearchuk.org/cancerstats/types/bowel/***

Patients with left sided / rectal tumours (approximately 75%) typically present with a change of bowel habit, colicky abdominal pain and per rectum bleeding. Right sided cancer typically presents with anaemia, as the liquid nature of the faeces and the wider diameter of the colon make obvious tumour related symptoms unusual. Guidelines have been developed to classify those at high risk warranting urgent investigation based on change in bowel habit, rectal bleeding in the absence of anal symptoms, palpable abdominal or rectal masses, and anaemia [64].

It is recommended that patients with higher-risk symptoms should be fast-tracked either in special clinics or with urgent appointments in routine clinics. Patients so referred should be investigated with sigmoidoscopy (flexible or rigid) plus a high quality double contrast barium enema, or colonoscopy, or CT colonography. With the exception of patients with peritonitis who require emergency surgery, all patients with colon or rectal cancer should have pre-operative staging by CT scan to determine the local extent of the disease and the presence of lung or liver metastases. Patients with rectal cancer should also have MRI scans of the pelvis to stage the tumour and assess involvement of adjacent organs. Endorectal ultrasound scanning should be performed to assess T1 rectal cancers when local excision is being considered [65].

For the purpose of this summary so far colonic and rectal cancers have been considered as a single disease process, however it is debated whether they should be considered as separate entities. Differences exist with respect to age and gender dynamics as well at tumour progression and adjuvant treatments. However, there is a good correlation between cancer incidence rates for both the sites observed in different ethnic populations and the shared similar aetiology, type of precancerous

lesions as well as mode of spread all give evidence to them being two variants of the same malignant process.

### 1.1.6 Treatment options

#### *1.1.6.1 Surgery*

Radical resection of a colonic tumour along with the appropriate vascular pedicle and accompanying lymphatic drainage is the most appropriate operation to gain local control [66]. This approach is appropriate for 80-90% of patients with localised colon carcinoma. As surgery is the only definitive curative treatment, technique is important to ensure a good outcome. Adequate lymphadenectomy is critical. In addition to the therapeutic benefits of preventing local progression and subsequent development of symptoms due to mesenteric recurrence, lymphadenectomy is critical in the staging of patients with colon carcinoma. In colon cancer, the status of recovered lymph nodes is one of the main parameters used for adjuvant therapy recommendations.

#### *1.1.6.2 Adjuvant treatments*

At present, only chemotherapy and radiotherapy have an established role as adjuvant treatment for colorectal cancer. Chemotherapy is discussed in section 1.2.

#### *1.1.6.3 Radiotherapy*

Radiotherapy is a potential adjuvant treatment only for those tumours localised in the rectum. There is unequivocal evidence that neo-adjuvant radiation reduces the risk of local recurrence in resectable rectal cancer [67, 68].

Patients with resectable rectal cancer should be considered for preoperative short course radiotherapy (25Gy in 5 fractions in 1 week), with surgery performed within 1 week of completion of radiation.

When local staging indicates that radiotherapy (with synchronous chemotherapy) would be appropriate to downstage the tumour and facilitate surgical resection, a dose of 45Gy in 25 fractions over 5 weeks, is recommended. If the addition of radiotherapy to surgery is deemed necessary for rectal cancer, it should ideally be given pre-operatively. However, in cases with well established predictive factors of local recurrence (e.g. evidence of tumour at the circumferential resection margin, mesorectal lymph node involvement and extramural vascular invasion), post operative radiotherapy and chemotherapy should be considered for patients who did not receive pre-operative radiotherapy. A dose of 45Gy in 25 fractions over 5 weeks with a planned boost dose of 5.4-9Gy in 3-5 fractions is recommended [65].

A planned radiotherapy volume using three or four fields given pre-operatively is recommended for rectal cancers as this results in less morbidity and mortality [67, 68].

### 1.1.7 Five Year Survival

The proportion of patients surviving for five years or more after their initial cancer resection is significantly influenced by the Dukes' stage of the original tumour, as shown in Table 1.5. Ten year survival rates hardly differ from these figures, as the vast majority of patients surviving for at least five years are considered cured from the disease.

***Table 1.5: Five-year relative survival of colorectal cancer patients (diagnosed 1996-2002) by stage at diagnosis, England.***

***Adapted from National Cancer Inteligence Network (NCIN). www.ncin.org.uk/publications/data_briefings/colorectal_cancer_survival_by_stage.aspx***

| Duke's Stage at diagnosis | Percentage of Cases | Five-year Relative Survival |
|---|---|---|
| A | 8.7% | 93.2% |
| B | 24.2% | 77.0% |
| C | 23.6% | 47.7% |
| D | 9.2% | 6.6% |
| Unknown | 34.3% | 35.4% |

## 1.2 Chemotherapy

Most chemotherapeutic drugs achieve their cytotoxicity by inhibiting mitosis, or inducing apoptosis, therefore exhibiting their greatest action against any rapidly dividing cells. These agents are largely non-selective, in that they impact on both enlarging tumours and other fast dividing cells, such as those responsible for hair growth and for replacement of the intestinal epithelium – leading to the side effect profiles which can be associated with these agents.

As chemotherapy affects cell division, tumours with high growth fractions are more sensitive to chemotherapy, as a larger proportion of the targeted cells are undergoing cell division at any time. Chemotherapy also affects well differentiated tumours more effectively since mechanisms regulating cell growth are usually still preserved. With succeeding generations of tumour cells, differentiation is typically lost, growth becomes less regulated, and tumours become less responsive to most chemotherapeutic agents. Near the centre of some solid tumours, cell division has effectively ceased, making them insensitive to chemotherapy. Another problem with solid tumours is the fact that the chemotherapeutic agent often does not reach the core of the tumour.

Chemotherapy may be administered with a curative intent or it may be used palliatively to decrease tumour load and increase life expectancy, a better toxicity profile generally being expected for the latter. Multiple agents are often used in combination therapy; the differing mechanisms of action minimise single-agent resistance and side effect profiles are generally reduced.

Chemotherapy may be used pre-operatively (neoadjuvant) to shrink the primary tumour, thereby rendering local therapy (surgery or radiotherapy) less destructive or

more effective. Alternatively it can be utilised post-operatively to treat any residual tumour cells and reduce recurrence rates, or palliatively in the treatment of advanced disease not amenable to surgical resection.

Performance status is used to ascertain an individual's capability to withstand a chemotherapy regime or to assess whether dose reduction is required. Only a fraction of the cells in a tumour die with each treatment (fractional kill), so the drug treatment is administered in cycles, with the frequency and duration of treatments limited by toxicity to the patient.

### 1.2.1 Fluorouracil (5-FU)

5-FU is a fluoropyridimine and an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen. The fluoropyridiamines were developed in the 1950s following the observation that rat hepatomas used the pyrimidine uracil, one of the four bases found in RNA, more rapidly than normal tissues, indicating that uracil metabolism was a potential target for antimetabolite chemotherapy [69]. The mechanism of cytotoxicity of 5-FU has been ascribed to the disincorporation of fluoronucleotides into RNA and DNA and to the inhibition of the nucleotide synthetic enzyme thymidylate synthase (TS). Fluorouracil rapidly enters the cell using the same facilitated transport mechanism as uracil [70]. 5-FU is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). These active metabolites disrupt RNA synthesis and the action of TS. The rate-limiting enzyme in 5-FU catabolism is dihydropyrimidine dehydrogenase (DPD), which converts 5-FU to dihydrofluorouracil (DHFU). More than 80% of administered 5-FU is normally catabolised primarily in the liver, where DPD is

abundantly expressed [71]. One approach to improving 5FU bioavailability and specificity has been to design 5-FU pro-drugs that avoid this DPD-mediated liver degradation. Capecitabine is an oral fluoropyrimidine that is absorbed unchanged through the gastrointestinal wall and is converted to 5'-deoxy-5-fluorouridine (5'DFUR) in the liver by the sequential action of carboxylesterase and cytidine deaminase [72]. 5'DFUR is then converted to 5-FU by thymidine phosphorylase and/or uridine phosphorylase, both of which have been reported to be significantly more active in tumour tissue than in normal tissue. This might account for the observed tumour-selective activation of capecitabine when compared to 5-FU [73].

### 1.2.2 Oxaliplatin

Oxaliplatin is a anti-neoplastic, platinum-based chemotherapeutic agent, complexed with a 1,2-diaminocyclohexane (DACH) and an oxylate group, to try and reduce toxicity and circumvent potential drug resistance (when compared with the earlier platinum compounds such as cisplatin). Within approximately two hours of intravenous administration, oxaliplatin is metabolised to platinum biotransformation products. It is thought these then interact with DNA to form both inter and intra-strand cross links, resulting in the disruption of DNA synthesis and leading to cytotoxic effects [74]. Specifically, Oxaliplatin reacts with DNA, forming adducts at the N7-position of guanine and to a lesser extent adenine [75]. This results in platinated intrastrand crosslinks between two guanines or a guanine adjacent to an adenine. Comparison of cisplatin and oxaliplatin binding to DNA by X-ray crystallography reveals that the DACH moiety of oxaliplatin and its bulky adduct creates a greater distortion in the major groove of DNA [76]. This elicits a distinct cascade of DNA repair processes and bypasses the DNA mismatch repair (MMR)

system [77]. At doses resulting in equivalent cytotoxicity, oxaliplatin forms fewer DNA adducts, suggesting oxaliplatin lesions are more lethal than cisplatin lesions. This is supported by observations of increased DNA strand breaks and apoptosis in cells treated with oxaliplatin when compared with cisplatin. The oxalate-leaving group is also important as it is more readily displaced relative to other platinums, contributing to high plasma protein binding and less renal clearance [78]. Resistance to oxaliplatin can occur via three mechanisms: i) diminished drug accumulation; ii) decreased adduct formation; and iii) increased DNA repair.

### 1.2.3 The role of chemotherapy in the management of colorectal cancer.

Chemotherapy has an increasing role in the management of colorectal cancer and has been a major contributing factor to the significant improvements in prognosis over the last two decades. Overall, adjuvant chemotherapy appears to confer a 5 – 10% improvement in absolute survival [79].

#### *1.2.3.1 Node positive disease:*

Large meta-analyses of historical data from randomised trials have demonstrated that post-operative systemic single-agent chemotherapy improves survival for patients with Duke's C tumours. The standard regimens were based on 5-flourouracil (5-FU) modulated by folinic acid (FA), and given for 6 months. Pooled data suggest that 5-FU/FA regimens can increase disease-free survival at 5 years from 42% to 58% and overall survival by as much as 13% (from 51% to 64%), when compared with surgery alone [80]. National guidance makes no distinction between colon and rectal cancer, and advises that all patients with node positive disease are offered chemotherapy, if they are deemed fit enough to tolerate its side effects.

More recently, oral forms of 5-FU (uracil-tegafur and capecitabine) have been licensed for this indication, on the basis of the results of two large randomised trials comparing their efficacy and safety with bolus 5-FU/FA in the postoperative adjuvant setting [81, 82]. Both confirmed that the oral drugs were at least as effective as the standard intravenous treatment. For example in the X-ACT study, after a median follow-up of 3.8 years, 35% of patients in the capecitabine arm had experienced disease recurrence or died, compared with 39% in the 5-FU/FA arm. With regard to survival, 80% and 77% of patients were alive in the capecitabine and 5-FU/FA arms, respectively, with no apparent differences in quality of life [82]. These agents are also associated with less toxicity and greater patient convenience. In April 2006 they received approval by NICE for adjuvant use.

There is overwhelming evidence of additional benefit from the use of combination therapy, specifically regimes based on oxaliplatin and 5-FU. Two phase III, randomised controlled trials that compared oxaliplatin containing regimens with standard treatment have been published [83, 84]. The first was the Multicenter International Study of Oxaliplatin / 5-fluorouracil and leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC) trial. This study included 2246 participants, 60% with stage III and the remainder with stage II colon cancer. The second was the National Surgical Adjuvant Breast and Bowel Project (NSABP C-07) trial, involving 2492 patients, 71% with stage III and the remainder with stage II colon cancer. In both trials the addition of oxaliplatin to 5-FU/FA, albeit administered via different regimes, led to a statistically significant reduction in rate of relapse when compared with 5-FU/FA monotherapy. Analysis of disease-free survival at 3 years showed a hazard ratio for recurrence of 0.77 (95% CI, 0.65 to 0.91) in the MOSAIC trial

(median follow-up 37.9 months), and 0.79 (95% CI, 0.67 to 0.93) in C-07(median follow-up 34 months). Toxicity was acceptable with low rates of persistent severe (greater than grade 1) neuropathy (<1%) and no excess of treatment associated fatality in the oxaliplatin containing arms. Oxaliplatin has been approved by NICE for this indication.

The challenge is now to determine for the individual patient which of these alternative approved treatments is the more appropriate for use as adjuvant therapy in node positive colorectal cancer. The benefit, in terms of improved likelihood of disease free survival from the use of oxaliplatin, should be set against the side effects and acceptability of the regimen. In general, a higher risk, otherwise fit patient should be offered oxaliplatin based adjuvant therapy as their risk of death from cancer significantly outweighs their risk of death from other causes.

#### *1.2.3.2 Node negative disease*

The magnitude of benefit for adjuvant chemotherapy in Duke's B tumours is smaller. Some studies of 5-FU based treatment have failed to demonstrate any benefit at all. Examples include the IMPACT B2 study, a pooled analysis of 1,016 patients with stage B2 colon cancer randomised to chemotherapy versus observation which showed no significant improvement in overall survival (OS 0.83, CI 0.72-1.07) [85]. In contrast, a grouped analysis of the National Surgical Adjuvant Bowel Project (NSABP) trials C-01 and 04, which included 2,151 Duke's B patients, concluded that the findings supported the use of adjuvant FU+LV as an acceptable therapeutic

standard in patients with Duke's B and C carcinoma of the colon [86]. The more recently reported UK QUASAR 1 study has also shown a modest benefit with bolus 5-FU/FA of around a 4% improvement in overall survival, and confirmed that this was cost-effective, especially in the under 70 age group. This data together suggests a small (5% or less) absolute increase in survival, for patients with Duke's B cancers, from adjuvant single agent chemotherapy. The MOSAIC trial included node negative patients, and a smaller but statistically significant incremental benefit to the addition of oxaliplatin was demonstrated.

### 1.2.4 Chemotherapy for advanced and metastatic colorectal cancer

A number of meta-analyses of palliative chemotherapy have shown improved survival with chemotherapy compared with best supportive care. The evidence indicates that early single agent chemotherapy prior to clinical deterioration for advanced disease improves survival by 3 to 6 months and either improves or maintains quality of life [87]. More recently combination chemotherapy with intravenous 5-FU plus either irinotecan or oxaliplatin has been shown to offer survival benefits in both first and second line situations. Current NICE guidance supports the use of all three of the active drugs (a fluoropyrimidine, oxaliplatin and irinotecan) and as such has deemed them cost effective [88].

Therefore, when offering multiple chemotherapy drugs to patients with advanced and metastatic colorectal cancer the following combinations of chemotherapy have NICE approval:

- FOLFOX (folinic acid plus fluorouracil plus oxaliplatin) as firstline treatment then single agent irinotecan as second-line treatment **or**

- FOLFOX as first line treatment then FOLFIRI (folinic acid plus fluorouracil plus irinotecan) as second-line treatment **or**
- XELOX (capecitabine plus oxaliplatin) as first-line treatment then FOLFIRI (folinic acid plus fluorouracil plus irinotecan) as second-line treatment.

Raltitrexed is only licensed for patients with advanced colorectal cancer who are intolerant to 5-fluorouracil and folinic acid, or for whom these drugs are not suitable.

### 1.2.5 Targeted Monoclonal Antibody Therapy

The most recent developments are with targeted monoclonal antibodies used in conjunction with chemotherapy. Bevacizumab (Avastin), an antibody to the vascular endothelial growth factor, has been shown to confer an additional benefit of 4.7 months in median survival, compared to irinotecan and 5-FU alone, in the first line setting [89]. Cetuximab (Erbitux), an epidermal growth factor receptor inhibitor, appears capable of overcoming drug resistance in second and third line situations [90]. Both these agents are licensed and at the time of writing are subject to a NICE appraisal. The preliminary guidance suggests that they are unlikely to be approved on the grounds of cost-effectiveness. It is hoped that the results of the numerous ongoing clinical trials with these and other molecularly targeted drugs, will demonstrate sufficient benefit to render them financially viable.

## 1.3 The role of genetics in colorectal carcinogenesis

Cancer cells are characterised by their ability to proliferate in defiance of normal controls and by their potential ability to invade other tissues. It has become clear during the revolution in cancer research in recent decades, that cancer is in essence a genetic disease. The regulation of cell growth, division and death is usually carefully controlled, with multiple safeguards to protect the cell from the potentially lethal effect of cancerous mutations. Therefore a series of genetic mutations are necessary within a given cell before an invasive cancer can develop. Alterations in three categories of genes are responsible for tumorigenesis: oncogenes, tumour suppressor genes and DNA repair genes.

Oncogenes are mutated forms of the proto-oncogene normally found within the cell. They render the gene constitutively active or active under conditions in which the wild type gene is not. Oncogenic activations can result from chromosomal translocations, gene amplifications or from subtle intra-genic mutations (missense mutations) affecting crucial residues that regulate the activity of the gene product. Oncogenic mutations are typically dominant in nature, requiring mutation to only one allele, to express the oncogenic phenotype.

Tumour suppressor genes are affected in the opposite manner, with genetic mutations leading to reduced activity of the gene product. This can occur as a result of nucleotide deletions, insertions or missense mutations leading to altered or truncated forms of the protein product. Mutations in both alleles are generally required to confer a selective advantage to the cell. This situation commonly arises when an intragenic mutation occurs, followed by a gross chromosomal loss of the other allele.

Oncogene and tumour suppressor gene mutations both drive the neoplastic process by increasing tumour cell number through the stimulation of cell division or the inhibition of cell death.

DNA maintenance genes are involved in the repair of DNA lesions incurred as a result of extrinsic cell damage or intrinsic replication errors and are therefore required to maintain the fidelity of DNA sequences. The first somatic mutation in an oncogene or tumour suppressor gene that causes a clonal expansion initiates the neoplastic process. Subsequent somatic mutations result in additional rounds of clonal expansion and thus tumour progression. Individuals with germline genetic mutations have a 'head start' on the neoplastic process as they only require one further allelic mutation to contribute to cancer formation. Such individuals often develop multiple tumours at an earlier age than individuals whose mutations have all occurred somatically.

## 1.4 Colorectal cancer - the genes / pathways involved

Cancer progression results from a series of genetic alterations leading to the progressive disordering of the normal mechanisms controlling growth. Colon cancer provides an excellent opportunity to study the stages of an evolving malignancy because tumours at various stages of development can be obtained for analysis. Early lesions may be by removed at colonoscopy or, in the case of more advanced tumours, tissue may be provided from an anatomical surgical resection.

In addition, the presence of hereditary syndromes associated with increased incidences of colon cancer (i.e. FAP and HNPCC) has allowed the concurrent study of inherited and sporadic genetic alterations to be considered. This has enabled the detection of shared mutations in affected families and therefore localisation and ultimately the identification of implicated genes.

Clinical and histopatholgical data suggest that most colon cancers arise from small pre-existing benign tumours (adenomas), which increase in size and dysplasia over several decades to eventually become large invasive and then metastatic neoplasms. In 1975, the term "adenoma to carcinoma sequence" was coined by Morson and colleagues at St. Marks in London to describe this histological progression [91]. In 1990, Fearon and Vogelstein published a model linking the accumulation of sequential genetic defects, with the clinical multi-step model of progression from adenoma to invasive carcinoma [92]. The main candidate genes responsible for the various stages were consequently identified as APC, K-RAS, DCC and p53.

Further work has indicated that this original model may be an oversimplification of colorectal carcinogenesis. Firstly the sequential model of mutation accumulation was questioned, with a study demonstrating that only 6.6% of tumours were found to contain mutations in APC, K- RAS and p53, with 38.7% of tumours containing mutations in only one of these genes. The heterogeneous pattern of tumour mutations suggested that alternative patterns to colorectal cancer may exist and that the widely accepted genetic model of cancer development may not represent the majority of colorectal tumours [93].

Hence, the Vogelstein model now appears to represent one pathway, amongst a number of complex and integrated routes, towards the development of colorectal cancer. In Figure 1.4, the classic Fearon and Vogelstein model is shown in red alongside other hypothesised routes towards colorectal cancer development.

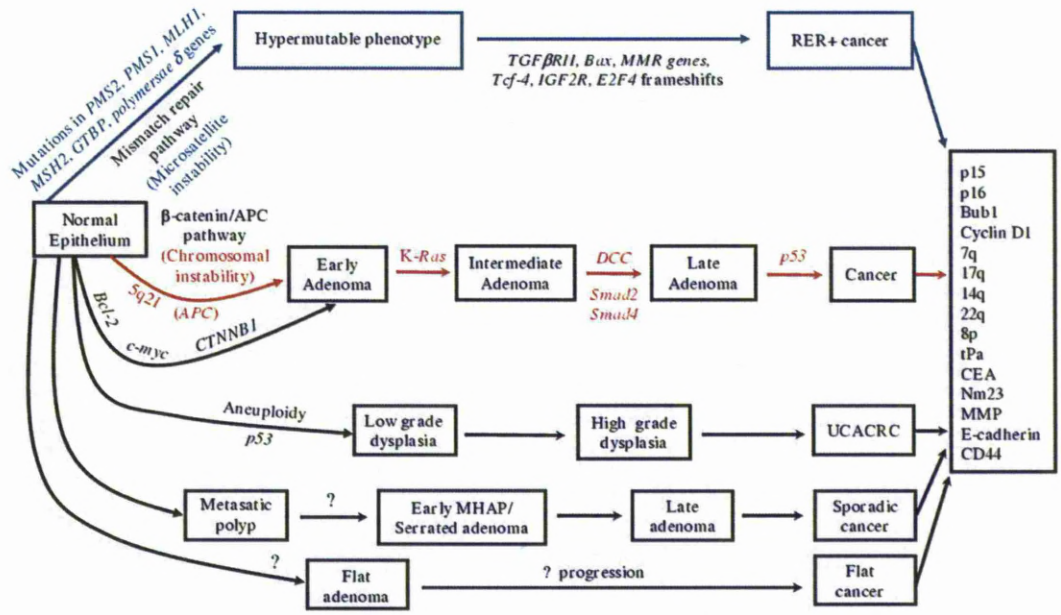



***Figure 1.4: Model for genetic alterations in the development of colorectal cancer. Reproduced from Narayan and Roy (2003) [94]***

Each of the key genes implicated in colorectal cancer pathogenesis will now be discussed in more detail through the following sections.

### 1.4.1 APC

The Adenomatous Polyposis Coli (APC) gene is a tumour suppressor gene located on chromosome 5q21. The APC gene product is a 312 kDa protein localised in both the cytoplasm and the nucleus [95, 96]. Somatic APC mutations are an early and perhaps rate-limiting event in the development of most adenomas and are present in approximately 70-80% of sporadic colorectal carcinomas [97-99].

The APC gene is abnormal in the germline of patients with familial adenomatous polyposis (FAP). This is an autosomal dominant syndrome in which hundreds of colorectal adenomas develop in affected person by the third or fourth decade of life, each of which can progress to carcinoma. FAP is found in about 1 in 12,000 individuals and accounts for approximately 0.5% of all colorectal cancers. Heterozygous germline mutations typically result in a truncated protein. Adenoma progression is initiated by a somatic loss of the second wild-type APC allele as predicted by the Knudson two-hit hypothesis for tumour suppressor genes. [100-103].

The APC tumour suppressor gene has been implicated in the regulation of cell-cell adhesion, cell migration, chromosomal segregation and apoptosis in the colonic crypt [72-74].

Although the APC protein more than likely has multiple critical cellular functions, the best established role for APC in the cancer process is as a major binding partner and regulator in the β-Catenin dependent Wnt signalling pathway. APC acts as a negative regulator of B-catenin. It stimulates B-catenin phosphorylation by GSK3 and its subsequent ubiquitination and degradation by the proteosome. The lack of functional APC protein or the deletion of its B-catenin binding domain, leads to over-activity of the B-catenin TCF7L2 transcriptional activation pathway and therefore promotes proliferation of the intestinal cell [104, 105], see Figure 1.5.

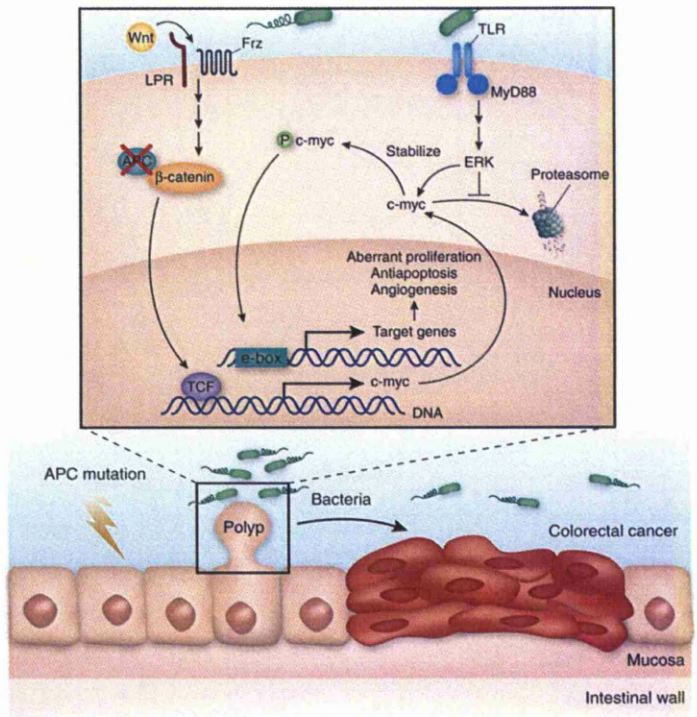



***Figure 1.5: The role of the APC pathway in colorectal cancer***

***Reproduced from Fukata and Abreu, Nature Medicine, 2010 [106]***

### 1.4.2 KRAS

KRAS is a member of a family of homologous oncogenes (the others being HRAS and NRAS) and is located on chromosome 12p12. It encodes a small 21kDa protein (p21ras). Approximately 40% of sporadic colorectal tumours are found to contain

mutations of the KRAS oncogene. The majority of KRAS mutations are gain-of–function missense mutations limited to codons 12, 13 and 61 [107].

Mutations of KRAS tend to occur at a relatively early stage in colorectal tumorigenesis. Whereas approximately 10% of adenomas less than 1cm contain the mutated, activated KRAS oncogene, this increases to 40-50% of adenomas greater than 1cm [108]. Ras proteins are thought to function downstream of multiple receptor tyrosine kinase (TRK) growth factors, such as the epidermal growth factor receptor (EGFR) family, as shown in Figure 1.6. This has become clinically relevant with the development of the targeted anti-EGFR monoclonal antibody Cetuximab. The results of the CRYSTAL trail demonstrated a significant improvement in the efficacy parameters of Cetuximab, when combined with the FOLFORI chemotherapy regime, in patients expressing wild-type KRAS. In contrast, no difference in efficacy was evident between treatment arms in the mutant KRAS subgroup [109].

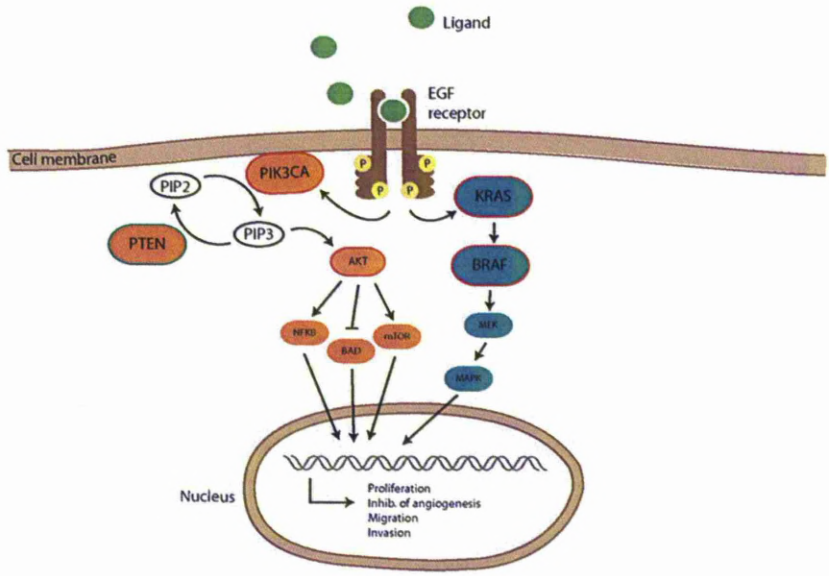



***Figure 1.6: The KRAS pathway***

***Reproduced from www.discoverymedicine.com***

Several studies have shown that patients who have a KRAS mutation in their colorectal tumour have a significantly poorer prognosis than those without a KRAS mutation [110, 111]. KRAS mutation is associated with an aggressive tumour phenotype and therefore detecting this mutation at an earlier disease stage may be of clinical importance.

### 1.4.3 The TGF-β Pathway

Loss of heterozygosity (LOH) of chromosome 18q is observed in 70% of CRCs; 50% of large, late-stage adenomas; and fewer than 10% of small, early stage adenomas [108]. Two chromosome 18q Tumour Suppressor Genes (TSGs) that are mutated in a fraction of colorectal cancers with 18q LOH are the SMAD2 and SMAD4 genes. Both genes encode proteins that function downstream of the TGF-β receptor complex, and the function of the SMAD2 and SMAD3 proteins is regulated by TGF-β-mediated receptor phosphorylation. Phosphorylation of SMAD2/3 allows the proteins to traffic to the nucleus and complex with the SMAD4 protein, and this complex can then bind to specific sequence elements and regulate gene transcription, as demonstrated in Figure 1.7. Mutations that inactivate SMAD4 are found in 10–15% of CRCs, and SMAD2 mutations are found in 5% of CRCs [112].

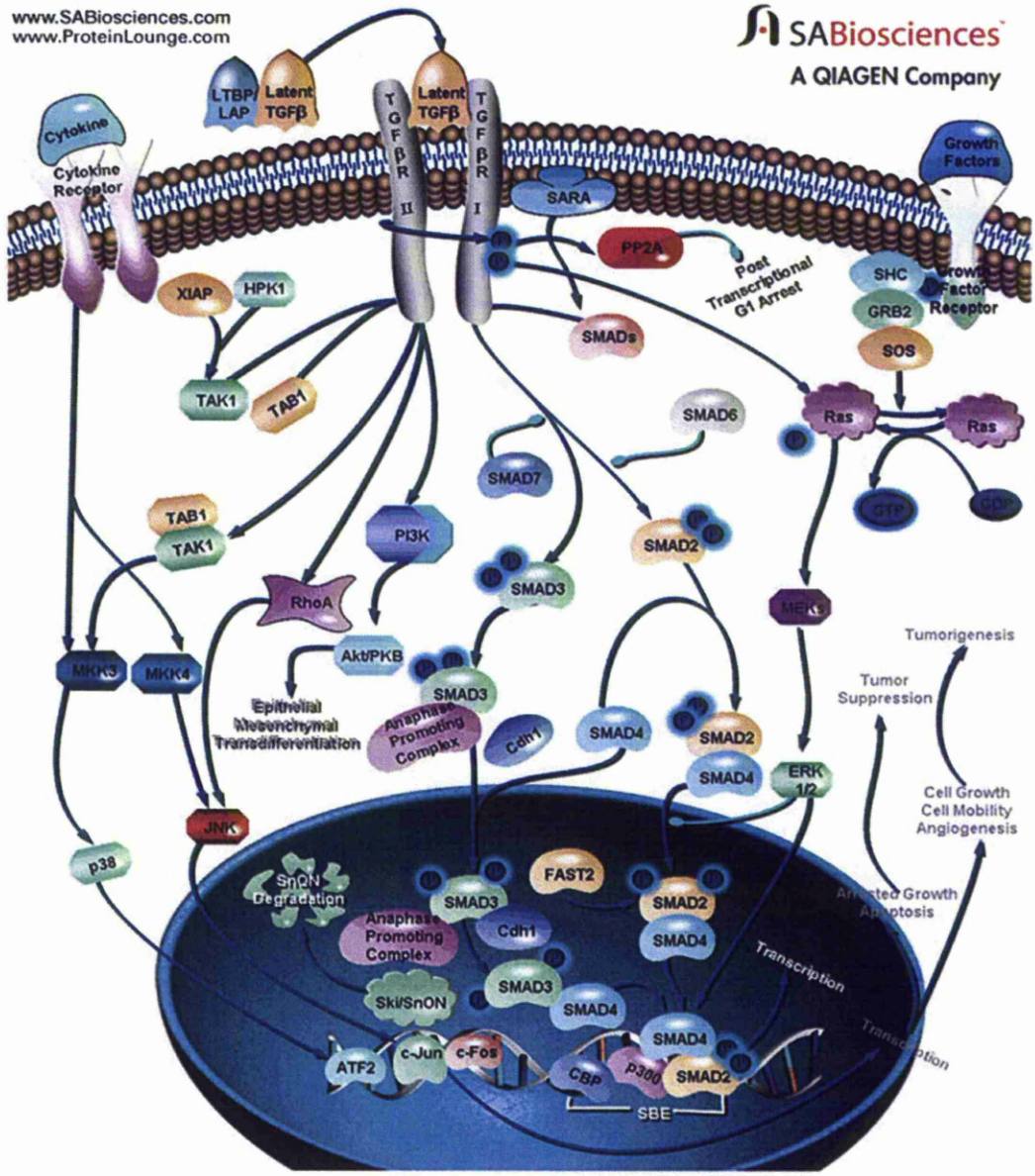



*Figure 1.7: The TGFβ pathway*

*Reproduced from www.proteinlounge.com*

Loss of 18q function appears to be implicated in the late stages of the pathogenesis of colorectal neoplasia, being infrequent in small adenomas but present in nearly 100% of hepatic metastases from colorectal primaries. Chromosome 18q LOH has also been associated with relatively poor prognosis in Duke's B staged disease [113, 114].

### 1.4.4 The Mismatch Repair (MMR) System, HNPCC and micro-satellite instability (MSI).

The Hereditary Non-Polyposis Colorectal Cancer (HNPCC) syndrome is an autosomal dominant condition which probably accounts for up to 5% of all colorectal cancers in developed countries. An excess of colon cancer and a defined spectrum of extra-colonic cancers, diagnosed at an early age constitute the clinical definition, which is strictly detailed in the 'Amsterdam criteria' [115, 116]. The syndrome arises as a result of germline mutation in one of several DNA mismatch repair genes (e.g. hMSH2, hMLH1, hPMS1, hPMS2, hMSh3, hMSH6).These genes control the repair of DNA base pair mismatches and are responsible for ensuring correct DNA synthesis during replication. Microsatellites are the result of base-pair substitutions and frame-shift mutations and result in short, tandemly repeated nucleotide sequences e.g. CACACACA. Microsatellites are found in a great number throughout the genome and owing to their repetitive nature, are prone to errors in replication. Microsatellite Instability (MSI) occurs when a germline microsatellite allele has undergone a somatic change in length. Nucleotide mismatches in micro-satellites are common but are usually rapidly corrected by the mismatch repair (MMR) genes. Hence defects in the MMR mechanism leads to widespread mismatch repairs, MSI and tumorgenicity. Approximately 15% of sporadic colon cancers result from MSI, rather than the classic chromosomal instability (CIN), with associated allelic mutations, loss of heterozygosity and aneuploidy as discussed thus far. In sporadic colon cancers the loss of expression of the MMR gene is often caused by aberrant methylation, and hence epigenetic silencing, resulting in deficient protein expression [117]. MSI associated tumours appear to have distinct clinical and pathological

features to those arising from CIN. They are more common proximal to the splenic flexure and occur more frequently in women [118]. Although the tumours tend to be larger and more poorly differentiated than their CIN counterparts, they have longer overall and cancer-specific survival, than stage matched patients exhibiting CIN [119]. Hepatic metastases from colorectal cancer rarely exhibit MSI [120].

### 1.4.5 Flat / depressed carcinomas

Flat and depressed lesions are defined as visible non-exophytic, flat and/or depressed mucosal lesions with a height less than half the diameter of the lesion [121]. These lesions are typically smaller in size than their polypoid counterparts, and are usually located on the right side of the colon [122]. Multiple reports from the Japanese literature suggest that flat and depressed adenomas are associated with a higher incidence of advanced histopathology, which includes high grade dysplasia and intra-mucosal adenocarcinoma, in relatively small tumours [123, 124]. The tumorigenicity of flat cancers remains uncertain but it is possible they follow a potentially different genetic pathway to those outlined for polypoid lesions. For instance, KRAS mutations occur at a lower frequency in flat lesions than in sporadic polypoid adenomas [125].

### 1.4.6 Other Genes Implicated in Colorectal Cancer

The MYC (also referred to as c-MYC) protein is a transcription factor that regulates genes with roles in cell-cycle progression, survival and various aspects of cellular metabolism in normal and neoplastic cells, as displayed in Figure 1.8. High copy

amplification of the *CMYC* gene is observed in approximately 5–10% of colorectal cancers, although moderate increases in MYC gene copy number and c-MYC protein expression may be found in more than 30% [126].

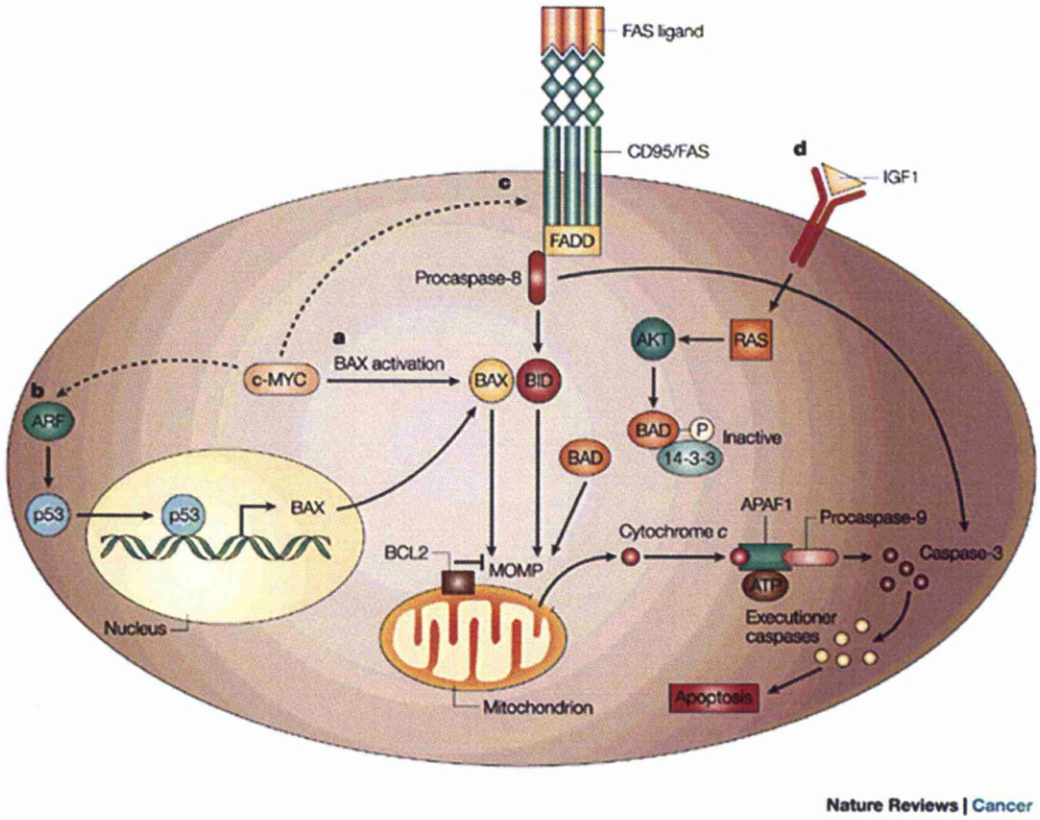



***Figure 1.8: The c-MYC pathway***

***Reproduced from Pelengaris et al, 2002[127]***

Many other genes that have been implicated in the tumorigenicity of colorectal cancer are shown in Table 1.6 alongside their estimated frequency of alterations:

***Table 1.6: Selected recurrent somatic mutations in oncogenes and tumour-suppressor genes***

***Adapted from Fearon (2011) [128].***

| Gene | Type of mutation | Estimated frequency of alterations |
|---|---|---|
| **Oncogenes** | | |
| *KRAS* | Point mutations (codons 12, 13, 61) | 40% (>75% of mutations are at codon 12) |
| *NRAS* | Point mutations (codons 12, 13, 61) | <5% |
| *PIK3CA* | Point mutations activating kinase activity | 15–25% |
| *BRAF* | Point mutations activating kinase activity (e.g., *V600E*) | 5–10% |
| *EGFR* | Gene amplification | 5–15% |
| *CDK8* | Gene amplification | 10–15% |
| *CMYC* | Gene amplification | 5–10% |
| *CCNE1* | Gene amplification | 5% |
| *CTNNB1* | Stabilizing point mutations and in-frame deletions near N terminus | <5% |
| *NEU* (*HER2*) | Gene amplification | <5% |
| *MYB* | Gene amplification | <5% |
| **Tumor-suppressor genes** | | |
| *TP53* | Point mutation, allele loss | 60–70% (>95% of point mutations are missense) |
| *APC* | Frameshift, point mutation, deletion, allele loss | 70–80% (nearly all mutations lead to truncated proteins) |
| *FBXW7* | Nonsense, missense, deletion | 20% |
| *PTEN* | Nonsense, deletion | 10% |
| *SMAD4* | Nonsense, missense, allele loss | 10–15% |
| *SMAD2* | Nonsense, deletion, allele loss | 5–10% |
| *SMAD3* | Nonsense, deletion | 5% |
| *TGFβIIR* | Frameshift, nonsense | 10–15% (>90% of MSI-H CRCs have mutations) |
| *TCF7L2* | Frameshift, nonsense | 5% (mutations in both MSI-H and MSS CRCs) |
| *ACVR2* | Frameshift | 10% (>80% of MSI-H CRCs have mutations) |
| *BAX* | Frameshift | 5% (often one allele in 50% of MSI-H CRCs) |

Abbreviations: CRC, colorectal cancer; MSI-H, high-frequency microsatellite instability; MSS, microsatellite stability.

## 1.5 p53

There is evidence for a malfunction in the p53 pathways in most cancers [129] and the mutation of the *TP53* gene that encodes *p53* is one of the most frequent genetic changes seen in malignant cells.

Consequently p53 is one of the most studied proteins in the whole of contemporary biology, with almost 62,000 citations on the 'PubMed' database (March 2012).

This massive interest in a single protein reflects the central role of p53 in the regulation of cell growth and differentiation and the frequency with which abnormalities of p53 function occur in human tumours.

### 1.5.1 The story so far....

It became clear in the early 1970's that RNA tumour viruses were able to cause cancer by their remarkable ability to capture and alter the expression of host genes that promote cellular growth. In 1979, David Lane and Lionel Crawford (Imperial College London) were studying the oncogenic activity of the small DNA virus *simian virus 40* (SV40). Studies of virally transformed cancer cells showed that the oncogenicity was due to the virally encoded T antigen. Lane and Crawford's paper showed that in virally transformed cancer cells the T antigen was tightly and specifically bound to a 53,000 molecular weight host protein that would become known as p53 [130]. Shortly following this, Linzer and Levine (Princeton) independently identified p53 through similar studies of virally transformed cells and also found the protein to be over-expressed in uninfected embryonic cancer cells [131]. In the same year, De Leo et al showed that the humoral response of mice to some methylcholanthrene-induced tumour cell line such as MethA was directed

toward the p53 protein [132]. Later, it was found that animals bearing several types of tumours elicited an immune response specific for p53 [133].

In the early eighties p53 was considered to be a likely oncogene, though its function and likely significance remained undiscovered. However, two important observations made during this period were that p53 is induced by DNA damage [134] and the identification of p53 gene alterations in virus-induced leukaemia's and tumour cell lines independently [135, 136].

A major paradigm shift occurred when it was demonstrated that wild-type p53 cDNA clones were in fact able to suppress transformation of rodent cells in culture while the point mutant version of p53 obtained from tumour cell lines were transforming. It was found that p53 point mutations were common in colorectal carcinomas and other tumours and in most cases one allele was mutated whereas the other was lost. In addition, inherited p53 mutations were found in the familial Li-Fraumeni syndrome (an autosomal dominant inherited syndrome in which affected individuals inherit a mutated p53 allele and are susceptible to a whole range of multiple sarcomas and carcinomas at a young age.) These findings in combination led to the recognition of p53 as a tumour suppressor gene [137].

In the following years, the induction of p53 in response to DNA damage was further substantiated and p53 was dubbed the "guardian of the genome" [138]. Studies of the effects of wild type p53 on human tumour cells showed that p53 could curb cell cycle progression and induce cell death by apoptosis. The generation of p53 null mice in 1992 and the demonstration of their dramatically increased tumour incidence further proved p53's tumour suppressive function [139]. Meanwhile, molecular studies revealed that p53 was a transcription factor with DNA binding specificity.

The first p53 target gene discovered was the cell cycle inhibitor p21/WAF1 in 1993, linking p53 with cell cycle arrest [140].

The identification of MDM2 as a p53 antagonist and critical regulator of p53 in 1997 led to the discovery that p53 stability is regulated partly through degradation in the proteasome [141, 142]. Since this time many further genes involved in the complex and closely regulated p53 pathway have been discovered and studied. Although p53 has been exstensively studied since its discovery approximately 33 years ago, there is still considerable work to be done before the potential production of efficient anti-cancer drugs that are based on targeting p53 in cancer cells.

### 1.5.2 Structure of p53

The p53 protein and its corresponding gene were named in reference of its mass of 53 kilodaltons. The gene is located on chromosome 17 at position p13 and is a phosphoprotein made of 393 amino acids. p53 is a modular protein with several regions with distinct, but inter-dependent functions, hence it has been described as having a four domain structure, as shown in Figure 1.9.

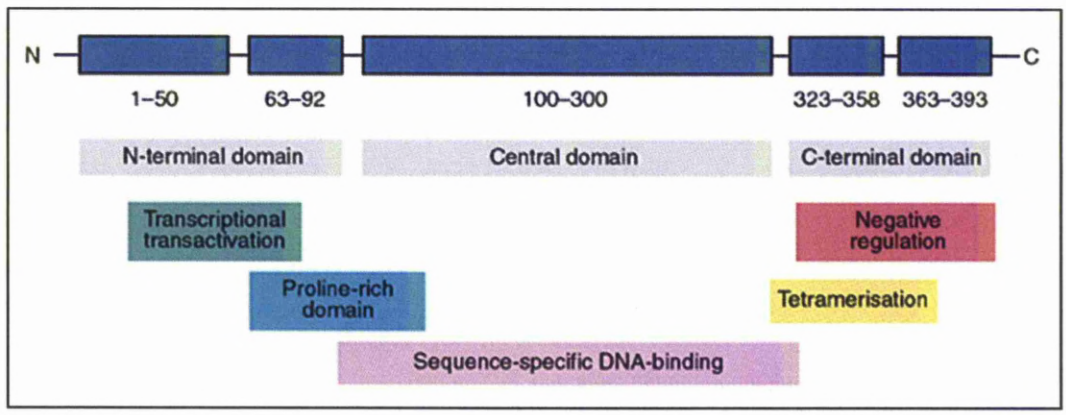



***Figure 1.9: p53 protein structure.***

***Reproduced from Anzola and Burgos, 2003 [143]***

Amino Terminus

The acidic amino terminus comprises the first 42 amino acids and constitutes a transcription activation domain. This area promotes expression of various downstream target genes in response to an activating stimulus. p53 possesses a hydrophobic interface in its N-terminal domain which interacts with the basal transcriptional machinery to promote up-regulation (or sometimes suppression) of p53 responsive genes through control of transcription of mRNA production. This region is also critically involved in regulating the stability and activity of p53 via interactions with proteins such as MDM2. Negative regulators of p53 mediated transcription, also target some of the same p53 amino acids critical to positive regulation of transcription activation.

$SH^3$ binding domain

Sandwiched between the transcription activation and the DNA binding domains (see Figure 1.9), is a region containing repeated proline residues, typical of a polypeptide that can interact with signal transduction molecules that contain a $SH^3$ binding domain, allowing p53 activity to be influenced. Through this domain p53 activity can be influenced by various signalling molecules including the c-abl oncogene.

Sequence specific DNA binding domain

The sequence-specific DNA-binding domain (DBD) of p53 is localised between amino acids 102 and 292. This region is a protease resistant and independently-folded domain, containing a $Zn^{2+}$ ion. The domain folds into a four-stranded and five

stranded anti-parallel *B* sheet that in turn is a scaffold for two α-helical loops that enable the region to interact directly with the DNA. More than 90% of the missense mutations of p53 occur in the regions of the gene encoding this domain [144]. Critical amino acid residues may be mutated resulting in defective contacts with the DNA and loss of the ability of p53 to act as a transcription factor. Alternatively, p53 mutations may structurally alter the conformation of the p53 protein in this region thereby disrupting its binding ability. The consequence of these events is the loss of the ability of p53 to specifically bind DNA in a sequence specific manner.

Tetramerisation domain

In order for DNA binding to occur, tetramerisation of the 3D structure of the protein must occur. Amino acid residues 324-355 are responsible for this configuration and are therefore determined the tetramerisation domain.

Carboxy-Terminus

The carboxy-terminus consists of 26 predominantly basic amino acids, which form a region with key regulatory properties. The p53 protein requires a structural change to activate it for sequence-specific binding to DNA. The non DNA binding or latent form of p53 can be activated by alterations in the carboxy-terminus, regulating the ability of p53 to bind to specific DNA sequences at its central DBD. For example, deletion of this domain, phosphorylation by a protein kinase at residue 378 and short single strands of DNA binding to this region all activate site-specific DNA binding by the central domain. The c-terminal domain helps promote the re-association of single to double stranded DNA or RNA. It also binds preferentially to DNA ends and

to internal deletions loops in DNA, as generated by replication errors that are then detected and fixed by mismatch repair processes [145].

### 1.5.3 Function

p53 functions as a transcription factor resulting in altered expression of a range of target genes involved in cell cycle inhibition, DNA repair, senescence and apoptosis. p53 protein levels are normally closely regulated to maintain appropriate control of cell growth and development. Mutations of p53 or factors leading to its inappropriate expression are associated with tumorigenesis. Normally there is a careful balance to be achieved between factors relating to increased synthesis and expression and those controlling the protein's degradation. p53 becomes activated when cells are stressed or damaged. Such cells pose a threat to the organism as they are more likely than undamaged cells to contain mutations and exhibit abnormal cell cycle control and therefore have a greater risk of becoming cancerous. The p53 protein shuts down the multiplication of stressed cells inhibiting their process through the cell cycle.

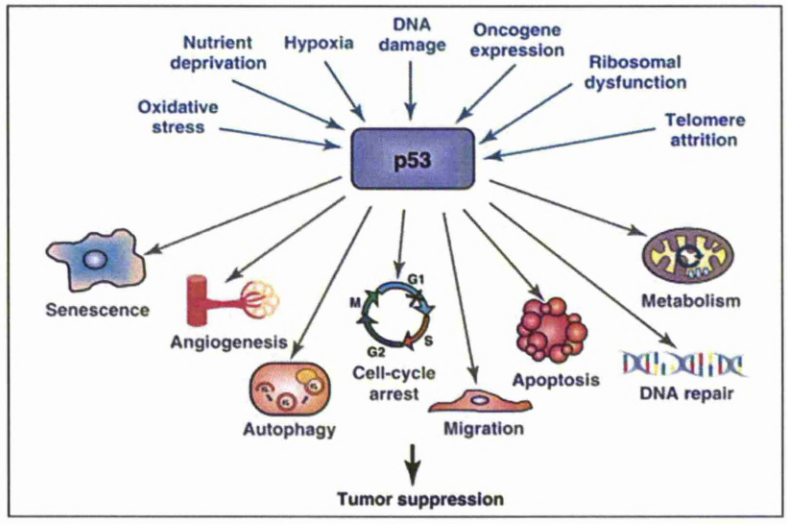



***Figure 1.10: p53 responds to a plethora of stress signals and regulates diverse responses. Reproduced from Bieging and Attardi (2011) [146]***

### 1.5.4 Activation and stabilisation

Regulation of p53 has been described at the level of transcription, translational and post-translational mechanisms and the latter can regulate conformational changes and various covalent and non-covalent modifications which alter levels and p53 activity. More than 36 different amino acids within p53 have been shown to be modified in various biochemical and cell culture studies (Figure 1.11). p53 protein levels in the cell are normally kept at a low concentration by its high turnover and thus has a relatively short half life in unstressed cells (~ 20 minutes) [147].

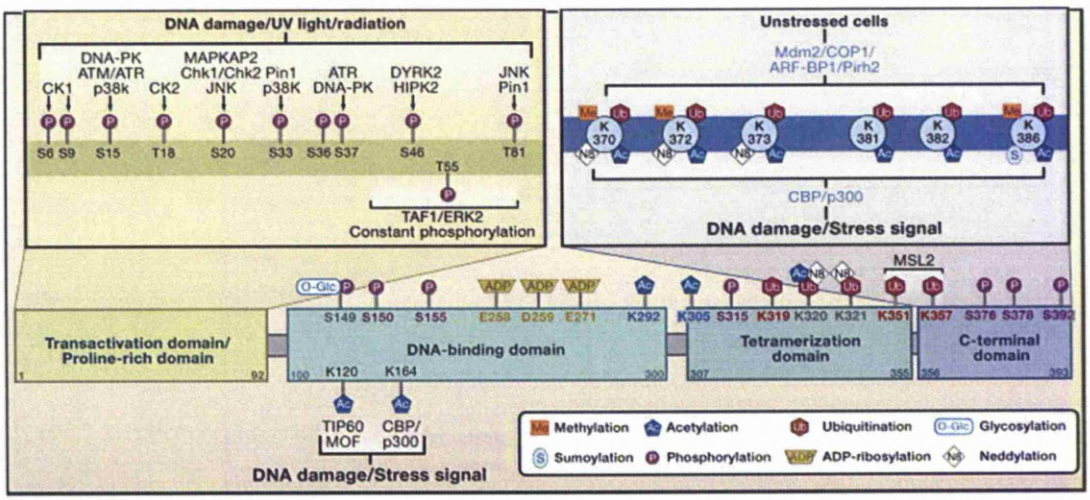



***Figure 1.11: Overview of p53 Posttranslational Modifications.***

***Reproduced from Kruse and Gu (2008) [148]***

*The major sites of p53 phosphorylation (P), ubiquitination (Ub), and acetylation (Ac) are shown with the corresponding major modifying enzymes and signals. Furthermore, additional phosphorylation and acetylation sites, as well as major sites of methylation (Me), sumoylation (S), neddylation (N8), glycosylation (O-Glc), and ribosylation (ADP), are indicated.*

Phosphorylation of p53 is classically regarded as the first crucial step of p53 stabilisation and this occurs rapidly, in response to various stress stimuli, to activate

p53. This modification by phosphorylation is initiated by a broad range of kinases, including ATM/ATR/DNA-PK and Chk1/Chk2. Phosphorylation of serine residues within the N-terminal p53 transactivation domain was among the first posttranslational modifications of p53 identified and has been extensively investigated in in vitro biochemical assays, in tissue culture studies, and recently by using site-specific knock-in animals. N-terminal phosphorylation at Ser15 (mouse Ser18) and Ser20 (mouse Ser23) have been generally thought to stabilize p53 by inhibiting the interaction between p53 and Mdm2 [149, 150].

The tight control of cellular p53 levels is primarily achieved through its ubiquitin-mediated proteasomal degradation [151]. Three independent studies identified the mouse double minute protein 2 (Mdm2) as the principle endogenous E3-ligase with high specificity for p53 [141, 142, 152]. The MDM2 protein leads to the mono-ubiquitination of p53: subsequently several copies of the small peptide ubiquitin are then attached to the protein. This ubiquitin chain acts a flag, labelling the protein for degradation.

Although the level of p53 is elevated in the absence of Mdm2, p53 is still degraded in the cells of Mdm2 null mice, suggesting the existence of alternative, Mdm2 independent pathways for p53 degradation in vivo [153]. The recently discovered E3 –ligases COP 1, Pirh2, Arf-BP1 and others, have clearly been shown to contribute to the efficient control of p53 levels in tissue culture and in in-vitro biochemical experiments [154-156].

There are multiple layers of regulation that connect Mdm2 function with p53 stability during stress responses. Several DNA damaging agents including UV, topoisomerase inhibitors and non genotoxic stresses such as hypoxia, induce a p53

response by specific inhibition of MDM2 transcription. A prominent physiological regulator of Mdm2 is the tumour suppressor ARF [157]. ARF interferes with the Mdm2-p53 interaction, thereby acting to stabilise and activate p53. The low steady-state levels of ARF in normal cells are dramatically induced upon oncogenic stress, which suppresses abnormal cell proliferation by triggering p53-dependent growth arrest or apoptosis [158].

The other prominent regulator of Mdm2 activity is MdmX. Like Mdm2, MdmX is a critical negative regulator of p53 [159].

Genetic evidence in humans suggests that ATM and CHK 2 are key players in the pathway of response to ionising radiation (IR). The ATM gene is mutated in the genetic disorder ataxia telangiectasia which is characterised by hypersensitivity to ionising radiation and predisposition to cancer. CHK 2 was found to be mutated in patients with Li-Fraumeni syndrome who do not carry a p53 mutation. Cells deficient for ATM or CHK 2 show a defect in their ability to synthesise p53 after exposure to IR, strongly supporting a role for these kinases in this damage response [160].

Transcriptional co-activators CBP/p300 and PCAF enhance p53 mediated transcription and are therefore considered important for p53 mediated growth arrest and apoptotic functions [161].

Increasing cellular levels of p53 alone do not initiate transcriptional activation. The extreme C-terminus of the protein controls the DNA binding sequence at the protein's core domain and hence the transcriptional activity. The folded 3-dimensional shape of the C-terminus usually inhibits DNA binding to the centre of the protein. A multitude of covalent and non-covalent modifications cause a

conformational change in this region allowing binding to occur. Phosphorylation, ubiquitination, methylation, sumoylation, neddylation and acetylation all target and modify the C-terminal of p53, thereby affecting the ability of p53 to bind DNA [149].

### *1.5.4.1 The p53 Response*

Cell Cycle inhibition

p53 acts as a transcriptional activator directly stimulating p21 expression. This in turn inhibits cyclin dependent kinases (CDKs). CDKs are key regulators of the cell cycle. For example, they ensure that DNA replication (S phase) follows on smoothly from the resting phase G1. Through its down regulation of various CDKs, p21 inhibits both the G1 to S and the G2 to mitosis transitions in the cycle. Cells depleted for p21 are deficient in both G1 and G2 arrest and in the coupling of DNA synthesis and mitosis. The same phenomenon is demonstrated in p53 null cells. Further potential mediators of G2 arrest regulated by p53 include GADD45, 14-3-3, sigmaB99, mcg10 and reprimo [162].

Apoptosis

Some cells in which p53 has become activated subsequently undergo programmed cell death. Numerous apoptotic genes that are transcriptionally activated by p53 have been identified, suggesting that the p53 apoptotic response is multi-faceted. The first apoptotic target of p53 identified was the Bax gene – a member of the BCL-2 family [163]. Transcription of the Bax gene has been shown in some human cells to be directly activated by p53 binding sites in the regulatory region of the gene. There are other pro-apoptotic members of the same family that have been shown to be

activated by p53, i.e. Noxa and PUMA (p53 upregulated modulator of apoptosis) [164, 165]. These proteins are all located within the mitochondria.

Apoptosis via loss of mitochondrial integrity may also be controlled by several genes coding for redox-controlling enzymes which were identified as p53 induced genes (PIGs) in a colon cell line undergoing p53 mediated apoptosis. It is hypothesised that these PIGs produce reactive oxygen species (ROS) which damage mitochondria and initiate apoptosis [166].

It has been suggested that p53 itself can localise to the mitochondria presenting a potential additional transcription independent way of mediating apoptosis.

p53 has also been implicated in the membrane death receptor induced pathway of apoptosis. DR5 / KILLER and FAS are two of the death receptors observed to be up regulated by p53 [167]. Activation of PIDD, a death domain containing protein, by p53, also induces apoptosis and is likely to function through the death receptor pathway [168].

Genetic Stability

p53 participates in DNA damage repair. It can bind primarily to damaged sections of DNA or can function indirectly via target genes that participate in 'nucleotide-excision' repair of DNA, chromosomal recombination and chromosomal segregation.

GADD45 binds proliferating cell nuclear antigen (PCNA) and could serve to inhibit DNA synthesis thus allowing DNA repair to proceed [169].

P53R2, a ribonucleotide reductase gene, is another transcriptional target of p53 that plays a role in DNA repair [170].

Inhibition of Blood Vessel formation

Angiogenesis into a malignant cell mass is essential for advanced tumour progression. p53 usually stimulates genes which suppress new blood vessel formation. Therefore cells in which p53 is inactivated would therefore be more likely to recruit new blood vessels, providing an important growth advantage at a late stage in tumour development. This corresponds with the stage of tumorigenesis when most natural p53 mutations occur.

## 1.6 The p53 pathway and its relevance to colon cancer.

In 1988 it was noted that allelic loss of the chromosome 17p region, containing the p53 gene, was frequently observed in polypoid colorectal cancers [171]. The first reports implicating p53 gene mutations in the development of colorectal cancer followed shortly thereafter [172, 173]. Early work showed that the growth of colorectal cancer cell lines in vitro could be suppressed by the introduction of wild-type p53, thus establishing the tumour suppressor properties of the gene [174]. Mutation of p53 is thought to increase the protein half-life and is therefore often associated with over-expression in the nucleus. In 1990, Rodrigues et al carried out immunohistologic staining of primary colorectal carcinomas with antibodies specific for p53. They demonstrated gross over-expression of the protein in about 50% of colorectal cancer cases. Conversely, benign adenomas were all negative for p53 over-expression [173]. The functional loss of p53 in colorectal cancer has been proposed as a late event in the transformation from adenoma to carcinoma [175].

An overview of fourteen studies, that each reported data on at least 50 colorectal cancer cases, estimated the frequency of p53 mutations at 45 % (1186 samples out of 2659) [176]. This is consistent within the range reported by other authors of 23-61 % [177-180]. Information on 1517 p53 mutations held in the UMD-p53 database [181, 182] indicates that 80% of the mutations are GC to AT single point transitions, occurring predominantly at CpG dinucleotides. These mutations are thought to arise by endogenous processes related to the deamination of 5-methylcystosine. Mutations in five hotspot codons (175, 245, 248, 273 and 282) account for approximately 43% of all p53 mutations in colorectal cancer [176, 181]. Three of these (codons 175, 248 and 273) contain a CpG dinucleotide. In 2002, Bazan et al confirmed that mutations

at codons 248 and 273 were the most frequently seen, with incidences of eleven and six percent respectively [183].

The prognostic significance of having a colorectal tumour containing mutated p53 has been the subject of intensive study and ongoing controversy. Several groups reported strong associations between p53 mutation and poorer prognosis in cohorts of more than 200 patients [177, 184], although both found the association was confined to distal tumours. However other large scale studies reported no association between p53 mutations and patient survival [176, 185, 186]. There has also been a lack of consensus regarding the significance of specific types of p53 mutation. In 1995, Goh and colleagues reported that mutations in the evolutionary conserved regions of p53 were associated with worse prognosis than those outside these domains [184]. In contrast, Kressner et al (1999) reported that mutations in the non-conserved region had the worst prognosis [180]. Borresen-Dale and colleagues (1998) found that mutations affecting the L3 zinc-binding domain had a significantly shorter cancer-related survival [177]. Different groups have suggested different prognostic significance depending on the ethnic group [187], site of tumour site in the colon [188] and stage of disease [189, 190]. The discrepancies in these findings are likely to represent the use of different mutation screening methods, as well as to the composition of the tumour series investigated, particularly with regard to tumour site, stage and the use of adjuvant therapies.

In order to try and resolve the issue of p53 mutations and prognostic implications in colorectal cancer, a large scale meta-analysis of all relevant previous studies, was performed by Munro et al in 2005 [191]. They performed a systematic review of 168

previous reports, incorporating a total of 18,766 patients. As with previous authors, they encountered problems with publication bias, heterogeneity of results and variability in assessment of p53 status and reporting of results. A trim and fill method was used to correct for publication bias and heterogeneity was minimised by using well-defined clinical subgroups of outcomes. Overall, patients with abnormal p53 were at increased risk of death: relative risk (RR) in studies using immunohistochemistry 1.32 (95% confidence interval, 1.23-1.42) and with mutation analysis 1.31 (95% confidence interval, 1.19-1.45). The adverse impact of abnormal p53 was greater in patients considered to have lower baseline risk of dying. That is, when taking the baseline median risk of death of 35%, and creating two arbitrary groups either side of this as good or bad prognosis patients, those considered to have good prognosis had a RR of death of 1.63 (95% c.i 1.40-1.90) and those with poor prognosis RR 1.04 (95% c.i. 0.91-1.19). Munro concluded that abnormalities in p53, whether assessed immunohistochemically or by sequence analysis, appear to be of no value in predicting response to 5FU-based chemotherapy. However, rectal tumours containing proven mutations in p53 are less likely to respond to radiation, or chemo-radiation, than rectal cancers without evidence of mutant p53.

The TP53 colorectal cancer international collaborative study on the prognostic and predictive significance of p53 mutation was also published in 2005 and refuted some of Munro's findings [192]. This study included data from a total of 3,583 colorectal cancer patients (from 17 different countries) with information on p53 gene mutation status. A significantly higher frequency of mutations ($P < .001$) was found in distal colon and rectal tumours (both groups, 45%) compared with proximal tumours (34%), confirming the findings of previous studies [176]. p53 mutations were

associated with lymphatic invasion in proximal tumours and showed trends for association with advanced Duke's stage (all sites) and with lymphatic (rectal tumours) or vascular invasion (distal and rectal tumours).

Previous in vitro studies had shown that disruption of p53 causes colorectal cancer cells to be more resistant to the apoptotic effects of FU [193]. The Russo et al. study found that colorectal cancer patients with wild-type p53 have significantly better survival when treated with chemotherapy compared with those treated with surgery alone, regardless of tumour site. In contrast, for patients with mutated p53, only those with proximal colon cancers showed significantly better survival when treated with chemotherapy compared with those treated by surgery alone. The results suggest that use of chemotherapy can influence survival depending on p53 mutation status; though this may be dependent on tumour site. Previous studies showing site-related differences in the frequency of p53 mutations and other genetic or epigenetic alterations have also suggested that these findings could translate into differential survival benefits from chemotherapy [194, 195].

The role of p53 in the cellular response to oxaliplatin has also been investigated using various *in vitro* models [196, 197]. These studies showed that human colorectal cancer cell lines lacking functional p53 were generally more resistant to 5-FU and oxaliplatin than the p53 wild-type cell lines analysed and that 5-FU and oxaliplatin sensitisation to Fas-mediated apoptosis required wild-type p53. Furthermore a retrospective clinical study in 2010 showed that p53 expression altered disease free survival when adding oxaliplatin to the adjuvant treatment regime of stage III colon cancer patients [198].

## 1.7 Gain-of-function mutations of p53.

p53 mutations have been linked to increased cell growth [199], enhanced tumourgenicity [200] and poor prognosis in a wide variety of tumour types. Disturbances in spindle checkpoint mechanisms [201] and increased tumour invasiveness [202] have also been demonstrated. It has been shown that p53 mutational status adversely affects cellular responses to chemotherapeutic agents when compared with cells lacking any detectable p53.

Unlike other tumour suppressor genes, whose inactivation occurs mainly by deletions, p53 is a frequent target of single nucleotide missense mutations, accounting for approximately 74% of all p53 mutations in human cancers [203]. Contrasting with wild-type p53, which under normal conditions has a short half-life curtailed when it is targeted by Mdm2 for degradation, mutant p53 proteins are outside this negative feedback loop and accumulate to high levels in cancer cells [204]. The large percentage of missense mutations, combined with the elevated level of expression of the tumour-derived p53 mutants, suggests that mutant proteins perform a vital oncogenesis role and are therefore selectively over expressed [205]. The p53 point mutations primarily occur in the conserved central region of the gene, coding for the DNA binding core domain (DBD). There are two classes of p53 DBD mutants: conformational and contact. The conformational mutants alter the global structure of the protein whilst the contact site residues bind directly with the DNA helix and accordingly are important for the transcriptional activation function of p53. Mutations in virtually every amino acid in this region (containing approximately 200 in total) have been documented, with some 'hot spot' mutations occurring at a markedly higher frequency than others. The resulting protein products are full-

length, with a single amino acid change, and are considered unable to recognise wild-type p53 consensus DNA binding sites [206].

### 1.7.1 Dominant – negative inhibition of wild type p53 function

It was originally hypothesised that the poor outcome associated with p53 mutations resulted from functional inactivation of the wild type p53 allele in a heterozygous genotype – so called dominant–negative inhibition. The proposed methodology behind this loss of tumour suppressive function was based upon the tetrameric nature of the p53 protein. It was felt that hetero-tetramers consisting of combinations of mutated and wild type proteins, may lead to abolition of the DNA-binding capacity of the whole complex. Tetramisation of the wild-type protein is mediated by the oligomerization region (residues 319 – 360). This region is fully functional in core domain mutants. Interestingly the 273 (R to H) mutants, which seems to retain almost all of their wild type conformation (98% folding of wild-type p53) has a very weak dominant-negative activity [207].

However, despite possible dominant-negative function of missense p53 mutants, in approximately 50% of human tumours harbouring such mutations, the remaining wild-type allele is mutated or lost, suggesting that complete loss of normal p53 promotes tumorigenesis further [208]. It has long been shown in knockout mouse model studies, that homozygous p53 null genotype is associated with tumour development, as anticipated with loss of tumour suppression function. However the tumour spectrum is fairly restricted, to primarily sarcomas and lymphomas, with carcinoma formation being rare [139]. In contrast, transgenic strains that produced over-expression of mutant p53, from exogenous promoters, resulted in accelerated tumour development with an increased incidence of carcinomas [209-212].

These findings lead to the theory that mutant p53 may also actively promote tumorigenesis independent of wild-type p53 function. This phenomenon became known as the "gain-of-function" model. The hypothesis being that p53 mutants can transactivate or repress a range of specific target genes, and the action of these genes mediate the various oncogenic effects of these mutants.

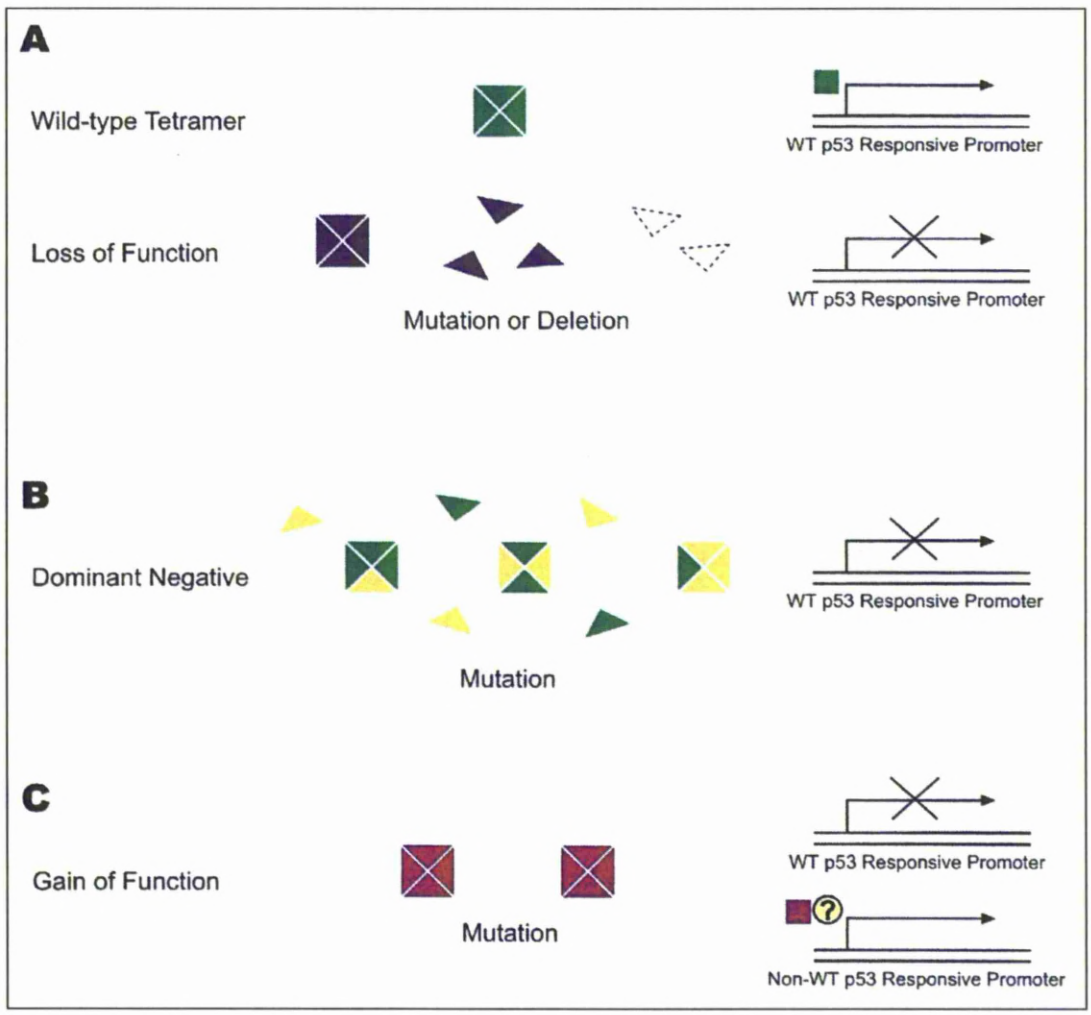



***Figure 1.12: Proposed mechanisms for the role of p53 mutations in tumorigenesis.***

***Reproduced from Cadwell and Zambetti, 2001 [213].***

*A - Loss of function. The wild-type p53 tetramer (green) is transcriptionally active in response to cellular stresses and induces the expression of downstream targets. An inactivating mutation (purple) or deletion of p53 (purple dotted line) results in a complete loss-of function that eliminates the p53 mediated stress response. B – Dominant-negative. Some p53 mutants (orange) oligomerise with wild-type p53 (green) and inhibit transcriptional activity. C – Gain-of-function. Other p53 mutants (fuchsia) possess new functions not shared by wild-type p53.*

### 1.7.2 The mouse models of mutant p53 gain of function

In 2004, two independent research groups published data that added considerable weight and credibility to the "gain-of-function" properties of mutant p53 proteins. To ascertain the physiological effects of p53 point mutations, transgenic mouse models were created with mutated p53 alleles engineered into the endogenous p53 locus. This allowed direct comparison of p53 heterozygotes (+/-) and p53 null (-/-) mice, with those containing p53 mutant alleles (M/-,M/-) expressed from endogenous promoters, under physiological conditions. These experiments improved on previous studies in which strong promoters had driven expression of the mutant transgene to very high levels, thus not accurately mimicking the occurrence of p53 mutations in human tumours.

Tyler Jacks group engineered two conditional point mutant p53 alleles into the endogenous murine locus, encoding the contact mutant $p53^{R270H}$ and the structural mutant $p53^{R172H}$. These correspond to the human codons 273 and 175 and represent two of the three most commonly mutated codons in human cancer. They found that M/- mice develop tumour spectra distinct from p53 null mice. Since the effects of any dominant-negative action had been negated, the increased incidence of carcinomas, adenomas etc. were deemed to result from a gain-of-function action of the mutant protein. The M/+ mice were also found to provide a more appropriate model to recapitulate the human familial Li-Fraumeni syndrome than do p53 +/- or -/- mice. They found that $p53^{R270H}$ mice developed primarily carcinomas whereas the highest tumour incidence in the $p53^{R172H/+}$ group were osteosarcomas, indicating that different p53 point mutations may confer subtly different functions. Tumour cells derived from the mutant p53 mice were discovered to respond to shRNA mediated

knockdown of their mutant p53 with a reduced rate of proliferation. This data in combination provided strong support for the protumorigenic effects of point mutant p53 [214].

Lozano's group published a very similar study with a further knock in p53R172H mutant. They found increased rates of metastases in the heterozygous mutants compared with the +/- mice but did not see the variation of tumour spectra demonstrated in the Tyler Jacks paper. They concluded this may result from the differing mouse strains utilised in the two experiments [215].

Both groups studied the interaction of mutant p53 with its two family member proteins p63 and p73, since it had been previously suggested that down regulation of these proteins may provide a mechanism for the gain-of function [216, 217]. Independently each team demonstrated that mutant p53 co-immunoprecipitated with p63 and p73. Levels of both proteins were low in the mutant tumour cell lines and an increase in p63 and p73 target gene expression occurred following knock down of mutant p53 with siRNA. Further work by Flores and Jacks involved the generation of p53+/- p63+/- and p53+/- p73+/- combination mice to further analyse the role of p63 and p73 in tumorigenesis. Mice heterozygous for mutations in both p53 and p63 or p53 and p73 displayed higher tumour burden and metastasis compared to p53+/- mice [218]. Hence strengthening the argument that p53, p63, p73 interactions may have an important role to play in p53 gain-of-function.

### 1.7.3 Mechanisms of gain-of function (GOF)

The interaction of Mutant p53 with p63 and p73.

A pivotal GOF mechanism is the ability of common p53 mutants to bind and inactivate p53 family members' p63 and p73. There is a substantial amount of data to support the role of the transactivation-potent variants of p63 and p73 in suppressing tumorigenesis. Analysis of mouse models demonstrated that p63 and p73 can partially compensate for deletion of *Trp53* as *Trp53*+/– *Trp63*+/– mice and *Trp53*+/–*Trp73*+/– mice have reduced survival and increased metastatic rate compared with *Trp53*+/– mice (Flores et al 2005). Therefore, inhibition of p63 and p73 function is considered a key mechanism for mutant p53 gain of function [219]. Evidence supporting this notion has come from the recently developed knock-in mouse model in which p53R172H was shown to bind p63 and p73 in tumour derived cell lines, consequently inhibiting their abilities to induce cell-cycle arrest and suppress focus formation [220]. The capacity of different p53 mutants to bind p73 has been shown to be significantly influenced by the site of mutation as well as by the single nucleotide polymorphism at codon 72 [221]. The p73 binding capacity is correlated with the ability of p53 mutants to protect cells from chemotherapeutic agents, and, accordingly, with less favourable response to chemo-radiotherapy in patients with head and neck cancer [221]. Therefore, targeting the interaction of mutant p53 with p63 and p73 seems a promising strategy for cancer therapy. Short peptides that disassemble p53R175H from p73 restore the activity of p73 and re-sensitize cells that harbour mutant p53 to chemotherapy [222]. Similarly, the small molecule RETRA, which interferes with mutant p53–p73 interaction, hinders the

growth of cells that express mutant p53 and their ability to form tumours in mice [223].

Transcriptional regulation by mutant p53

Modulation of gene transcription by mutant p53 is well documented as an important gain of function mechanism. The earliest evidence emerged with the demonstration that a functional transcription-activation domain of p53 is required for mutant p53 dependent activation of MDR1 and protection from drug induced apoptosis. Since then, the transcriptional regulation of numerous genes has been implicated in mutant p53 gain of function: a summary of which is detailed in Table 1.7.

*Table 1.7: Selected list of genes that are transcriptionally regulated by mutant p53.*

*Reproduced from Brosh and Rotter 2009 [224].*

| Phenotypic effects | Corresponding gene(s) | Effect |
|---|---|---|
| Enhanced proliferation | NF-Y target genes (such as *CCNA*, *CCNB2*, *CDK1*, *CDC25C*) | Upregulated |
| | MAP2K3 (mitogen-activated protein kinase kinase 3) | Upregulated |
| | *MAD1L1* (MAD1 mitotic arrest deficient-like 1) | Upregulated |
| | *FOS*, also known as *c-fos* | Upregulated |
| | *PCNA* (proliferating cell nuclear antigen) | Upregulated |
| | *MYC*, also known as *c-myc* | Upregulated |
| | *E2F5* | Upregulated |
| | *ASNS* (asparagine synthetase) | Upregulated |
| | *ARHGEF2* (Rho/Rac guanine nucleotide exchange factor (GEF) 2; also known as GEF-H1) | Upregulated |
| | *ID2* (inhibitor of DNA binding 2) | Downregulated |
| | *MCM6* (minichromosome maintenance complex component 6) | Upregulated |
| | *IGF1R* (insulin-like growth factor 1 receptor) | Upregulated |
| | *CXCL1* (CXC-chemokine ligand 1; also known as *GRO1*) | Upregulated |
| Inhibition of apoptosis and/or chemoresistance | *EGR1 (early growth response 1)* | Upregulated |
| | *ATF3 (activating transcription factor 3)* | Downregulated |
| | *LGALS3 (lectin, galactoside-binding, soluble; also known as Galectin-3* | Upregulated |
| | *FAS*, also known as *CD95/APO-1* | Downregulated |
| | *MST1 (macrophage stimulating 1; also known as MSP)* | Downregulated |
| | *BCL2L1*, also known as *bcl-xL* | Upregulated |
| | *DHCR24* (24-dehydrocholesterol reductase; also known as seladin-1) | Upregulated |
| | *NFKB2 (nuclear factor-κB2)* | Upregulated |
| | *ABCB1* (ATP-binding cassette, sub-family B (MDR/TAP), member 1; also known as MDR1) | Upregulated |
| | *IGF2 (insulin-like growth factor 2)* | Upregulated |
| | *BAG1* (BCL2-associated athanogene) | Upregulated |
| | *DUT (deoxyuridine triphosphatase; also known as dUTPase)* | Upregulated |
| Other effects | *TGFBR2* (transforming growth factor, beta receptor II) | Downregulated |
| | ARHGDIA (Rho GDP dissociation inhibitor (GDI) α) | Upregulated |
| | RANGAP1 (Ran GTPase activating protein 1) | Upregulated |
| | *PXN (paxillin)* | Upregulated |
| | *KIF20A (kinesin family member 20A)* | Upregulated |
| | *ALOX15 (arachidonate 15-lipoxygenase)* | Upregulated |
| | ribosomal proteins *RPL37*, *RPLP1*, and *RPS2* | Upregulated |
| Limitless replication | *TERT (telomerase reverse transcriptase)* | Upregulated |
| Invasiveness, inflammation and angiogenesis | NFκB target genes (such as *CXCL1*, interleukin 1β (*IL1B*), *IL6*, *IL8*, *MMP3*) | Upregulated |
| | *WISP2 (WNT1 inducible signalling pathway protein 2; also known as cyclin 5)* | Upregulated |
| | *VEGFA (vascular endothelial growth factor A)* | Upregulated |

*Rotter V. *et al.*, unpublished data. CDC25C, cell division cycle 25C; CDK1, cyclin dependent kinase 1; MMP3, matrix metalloproteinase 3.

Mutant p53 stabilisation.

Efficient GOF action by mutant p53 requires elevated levels of the mutant protein in the affected cell. Mutant p53 is not intrinsically stable; rather changes that occur in tumour cells result in this stabilisation. Hence, mutant p53 protein levels are low in

mutant p53 knock in mice but increase substantially in a fraction of tumours that emerge in such mice [220].

The simplest explanation for mutant p53 stabilization is that mutant p53 lacks the ability to transactivate wtp53 target genes. The Mdm2 gene is a classical positive transcriptional target of wtp53, and this drives a negative feedback loop that helps maintain wtp53 levels very low in unstressed cells. However, mutant p53 fails to transactivate the Mdm2 gene. Hence, Mdm2 protein levels are likely to be rather low in cells that express only mutant p53. Indeed, ablation of endogenous Mdm2 in mutant p53 knock-in mice leads to a substantial increase in endogenous mutant p53 levels [225]. Importantly, this results in an earlier age of tumour onset and a GOF metastatic phenotype.

Mutant p53 and Genomic instability

Human mutant p53 can increase genome instability by disrupting normal spindle checkpoint control, leading to accumulation of cells with polyploidy genomes [226]. Additional studies have revealed further manifestations of the enhancement of genomic instability by mutant p53, as reflected by higher mutation rates in the T-cell receptor of cells exposed to X-irradiation [227], increased frequency of centrosome amplification and aberrant mitoses in mouse mammary epithelial cells [228], as well as increased gene amplification in Saos2 cells [229]. This link was extended to an in vivo context, revealing that expression of the mouse equivalents of human "hotspot" p53 mutants results in tumours that exhibit a high degree of genomic instability, manifested by aneuploidy associated with aberrant centrosome amplification as well

as nonreciprocal chromosome translocations without evidence of telomere erosion [230].

The ability of mutant p53 to disrupt mechanisms that maintain cellular genome integrity might provide an appealing explanation for its impact on tumour progression, particularly in advanced stages of the disease in which gross manifestations of genomic instability are very frequent.

### 1.7.4 The relationship between mutant p53 expression, anti-apoptosis and response to adjuvant treatments.

Some p53 GOF mutants have an ability to confer on cells an elevated resistance to proapoptotic signals. In 1995 it was shown that mutant p53 can reduce apoptosis in leukemic cells [231]. Mutant p53 can also protect cells against apoptosis induced by growth factor deprivation [232]. The therapeutic efficacy of anti-cancer agents depends strongly on their ability to trigger apoptosis in target tumour cells [233]. Many physical and chemical DNA damaging agents used routinely in cancer therapy are potent p53 activators [234]. In 1999, Blandino et al studied the effects of exogenous mutant p53 transfection on the response to chemotherapeutic agents. Clonogenic survival assays revealed that cells over-expressing mutant p53 proteins became more refractory to the cytotoxic effects of chemotherapeutic drugs. Their findings suggested that over-expression of at least some forms of mutant p53, may directly enhance the resistance of tumour cells to anti-cancer agents and therefore that p53 gain-of function might contribute to failure of chemotherapy [235]. Increased resistance to $\gamma$ irradiation, doxorubicin and cisplatin was also shown in mouse cells expressing a murine mutant p53 isoform [236].

## 1.8 RNA interference

### 1.8.1 The History of RNAi discovery.

As is so often the case with major scientific breakthrough; the concept of RNA interference was discovered by a combination of fortunate accident and clever interpretation of unexpected resuls. The first observation of an RNAi type phenomenon was made by Napoli and Jorgensen in 1990 [237]. They were studying the anthocyanin biosynthesis pathway, which is responsible for the deep purple colouration of petunias. They introduced a chimeric Chalcone synthase (CHS) gene and hypothesised that its over-expression would lead to deep violet colouration, as the enzyme was felt to be the rate limiting step in the flavonoid pathway. Unexpectedly white petunias were produced, which correlated with a 50 fold reduction in endogenous CHS mRNA expression, suggesting that the transgene was somehow "cosupressing" the endogenous CHS gene. In 1992 Romano and Macino reported a similar phenomenon in Neurospora crassa (red bread mould), noting that the introduction of homologous RNA sequences caused "quelling" of the endogenous gene [238]. The phenomenon of RNA silencing was first documented in animals by Guo and Kemphues in 1995 [239]. They observed that the introduction of sense or antisense RNA to par-1 mRNA resulted in degradation of the par-1 message in Caenorhabditis elegans (nematode). Previously antisense was thought to function by hybridisation with endogenous mRNAs resulting in double stranded RNA (dsRNA), which either inhibited translation or was targeted for destruction by cellular ribonucleases. However, when sense par-1 transcript was used as a control, the message was still targeted for degradation.

A seminal paper offering explanation for this endogenous gene silencing came from Fire and Mello in 1998 [240]. They reasoned that the seemingly paradoxical finding of sense strand silencing made by Guo and Kemphues could have been due to the contamination of preparations of ssRNA by dsRNA resulting from the activity of bacteriophage RNA polymerases They indeed found that dsRNA was substantially more effective at producing interference than was either purified strand individually. Only a few molecules of injected dsRNA were required per affected cell, arguing against stochiometric interference with endogenous mRNA and suggesting that there could be a potent amplification component in the interference process. The observation of gene silencing being passed from parent to progeny led to the hypothesis that the effect may be mediated by a stable silencing intermediate. Further work on drosophila demonstrated that dsRNA was converted to shorter intermediates, small interfering RNA's (siRNAs), 21 -23 nucleotide lengths of RNA, capable of binding to their homologous target mRNA's leading to cleavage of the transcript. This seminal finding of the mechanism of RNA interference has recently resulted in Andrew Fire and Craig Mello being awarded the Nobel Prize for Medicine.

### 1.8.2 The significance of RNA interference.

RNA interference is an evolutionary conserved cellular defence observed in nearly every eukaryote studied thus far, and represents a unique form of post-transcriptional gene silencing (PTGS), initiated by dsRNA. In nature, RNAi is initiated when the host cell encounters a long dsRNA transcribed from an invading virus or from an endogenous source such as a mobilised transposon or an inappropriately transcribed

sequence. The discovery that delivery of siRNAs to mammalian cells caused sequence-specific gene silencing by RNAi has revolutionised the study of loss of gene functions. The ease with which genes can be silenced via the RNAi pathway has led to the generation and screening of genome-wide siRNA libraries in many organisms [241].

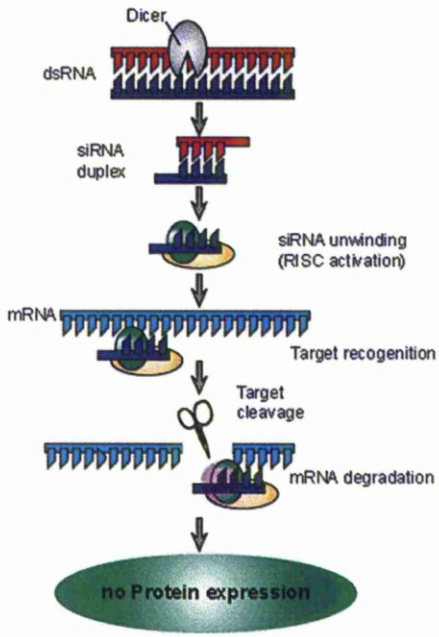
More recently a new class of regulatory, non-coding microRNAs (miRNAs) have been identified which has broadened the implications on RNAi even further. miRNAs are endogenously produced, short double stranded RNA molecules that can regulate gene expression via RNA interference. These molecules can inhibit gene expression by mRNA degradation or by translational inhibition of target genes. MiRNA genes constitute approximately 1 – 5% of the predicted genes in the human genome and bioinformatics analyses suggest that up to 30% of human genes may be regulated by miRNA's [242]. It has been shown that they could act as tumour suppressors by inhibiting oncogenes, or function as oncogenes by inhibiting tumour suppressors. More than 50% of miRNA genes have been found to be localised in cancer-associated genomic regions or in fragile sites [243]. Studies have linked diseases to the loss of miRNA expression that may result from the deletion or the translocation of a chromosomal region. For instance, consistent down-regulation of miR-143 and mir-145 have been observed in advancing colorectal cancer.

### 1.8.3 1.7.3 RNA interference: Mechanism of action

The introduction of double-stranded RNA into cells, results in their recognition by an RNase III enzyme, known as dicer; which cleaves the RNA into 21-23 nucleotides

siRNAs. This step is bypassed by using synthetic siRNAs but is utilised when short – hairpin sequences are used for silencing, as these are cleaved intra-cellulary by the Dicer enzyme into siRNA molecules. In the early years of experimentation with siRNA, its usage was limited to flies, worms and plants, since the introduction of long dsRNA into mammalian cells elicits an interferon response that causes a general inhibition of translation, abrogating the specificity of RNAi. The finding that short dsRNA could silence genes, bypassed this problem and heralded the use of RNAi in mammalian cells.

Once cleaved by Dicer, the 21-23 nucleotide dsRNA consequently becomes incorporated into a multi-protein, RNA – induced Silencing complex (RISC). This ternary complex consists of the Dicer enzyme, along with two other proteins; Ago 2 and TRBP (the HIV trans-activating response RNA – binding protein). Upon formation of the complex, Ago2 cleaves the passenger strand of the siRNA duplex, which then allows the complex to associate with the target mRNA. The single-stranded siRNA in RISC guides sequence – specific degradation of complementary or near complimentary target mRNAs. RISC cleaves the target mRNA in the middle of the complementary region, ten nucleotides upstream of the nucleotide paired with the 5' end of the guide siRNA. The RISC complex catalyses hydrolysis of the target RNA phosphodiester linkage, yielding 5'phosphate and 3'hydroxyl termini on the mRNA fragments. The cleaved mRNA sequence is therefore not translated into a functional protein product and the target gene has been silenced.




**Mechanism of action of RNAi.**

**Double stranded RNA is introduced into a cell and gets chopped up by the enzyme dicer to form siRNA. siRNA then binds to the RISC complex and is unwound. The anitsense RNA complexed with RISC binds to its corresponding mRNA which is the cleaved by the enzyme slicer rendering it inactive. (http://www.bioteach.ubc.ca/MolecularBiology/AntisenseRNA/)**

*Figure 1.13: Mechanism of action of RNAi*

*Reproduced from www.bioteach.ubc.ac/MolecularBiology/AntisenseRNA*

### 1.8.4 RNA interference and p53.

In 2002, Martinez et al, utilised siRNAs targeted against wild-type and mutant p53 sequences. They performed their experiments on H1299, p53 null cells, transfected with either the wild type p53 sequence or the common R248W mutant form. They tested various siRNAs for their efficiency and ability to discriminate between wild-type and mutant p53. They found the most effective and specific mutant silencing occurred when the mutated base was the ninth nucleotide from the 5' end. They demonstrated that their WT-siRNA inhibited the expression of the WT protein whilst

having little effect on the mutant protein levels. Conversely their mutant siRNA showed high selectivity for the mutant protein over the wild type [244].

Olive et el (2004) utilised RNAi technology following their transgenic studies into p53 gain of function (see 1.6.4). They took cells from an osteosarcoma metastasis that arose from a R172H / - mouse and introduced a conditional lentiviral shRNA expressing construct, to knock down p53 in an endogenous setting. A proliferation assay confirmed that p53 shRNA expressing cells grew more slowly after suppression of mutant p53, than cells undergoing transfection with an empty control vector [214].

In 2007, Vikhanskaya et al. used siRNA against mutant p53 and also p73. They showed that silencing of mutant p53 expression resulted in up-regulation of p53-target genes in several human cancer cell lines, leading to reduced cellular colony growth due to the induction of apoptosis [245].

### 1.8.5 The use of RNA interference in colon cancer.

RNA interference has been used to study multiple aspects of the genetics involved in the tumourigenicity of colon cancer.

It has been demonstrated that siRNA mediated gene silencing of vascular endothelial growth factor (VGEF) in colon cells can produce a 67% decrease in cellular proliferation Hence it is suggested that RNA interference could provide a potential mechanism for anti-angiogenic tumour therapy in colorectal cancer [246].

RNA interference has also been utilised to study the implications of oncogenic Ras mutations on levels of apoptosis, in malignant colon cells undergoing treatment with 5 FU chemotherapeutic agents [247].

### 1.8.6 The pSUPER vector

In 2002, Brummelkamp et al published their first report of a new vector system which directed the synthesis of small interfering RNAs in mammalian cells. They designed a gene specific insert, such that it specified a 19 nucleotide sequence derived from the target sequence, separated by a short spacer from the same 19 nt sequence in reverse. The resulting transcript was predicted to fold back on itself forming a short hairpin RNA structure intra-cellularly. They demonstrated that siRNA expression by this vector caused efficient and specific down-regulation of gene expression, resulting in functional inactivation of the target gene. They also found that a single nucleotide mismatch in the target sequence abrogated the ability to suppress gene expression [248].

Brummelkamp et al went on to develop a retroviral delivery mechanism for this pSUPER vector and showed that it could be used to specifically and stably inhibit expression of the K-Ras oncogene in human tumour cells [248].

### 1.8.7 pSuper used to down regulate mutant p53

Bossi et al (2006) published a paper demonstrating the use of the pSUPER retroviral vector against mutant p53 expressing cells. They demonstrated knock down to 20 – 50% of original levels and showed that this resulted in a concomitant reduction in cell proliferation and improved cell death rates in response to chemotherapeutic agents [249].

### 1.8.8 The clinical applications of RNA interference

If RNAi is to provide future therapeutic applications then successful and safe *in vivo* delivery of siRNA sequences must be established. The most challenging issue is maintaining stability of the desired sequence until the active siRNA oligos reach their target cells. SiRNAs are unstable in the serum environment where they can be degraded by RNAase and are rapidly excreted in the urine if delivered systemically. Overcoming multiple tissue barriers and ensuring efficient endocytosis into the target cell are also essential to successful delivery. The use of cationic lipids and polymers as siRNA delivery carriers have been shown to help overcome some of these obstacles. Options for local delivery under consideration include: intra-tumoral, intra-muscular, intra-peritoneal and intra-vitreous [250].

Two phase I clinical trials have been published using intra-vitreous delivery of siRNA to down regulate VEGFR1 in the treatment of age-related macular degeneration [251, 252]. Both trials reported promising results with minimal adverse affects and no unwanted systemic delivery.

## 1.9 Hypothesis / aims

**Hypothesis** – Inhibiting mutant p53 in colorectal cancer cells may reduce malignant potential and augment response to adjuvant therapies.

**Aims:** To utilise RNA interference to achieve stable knock down of mutant p53 protein levels in a panel of colorectal cancer cell lines in culture and to consequently study the effects of this on cell proliferation and response to chemotherapeutic agents.

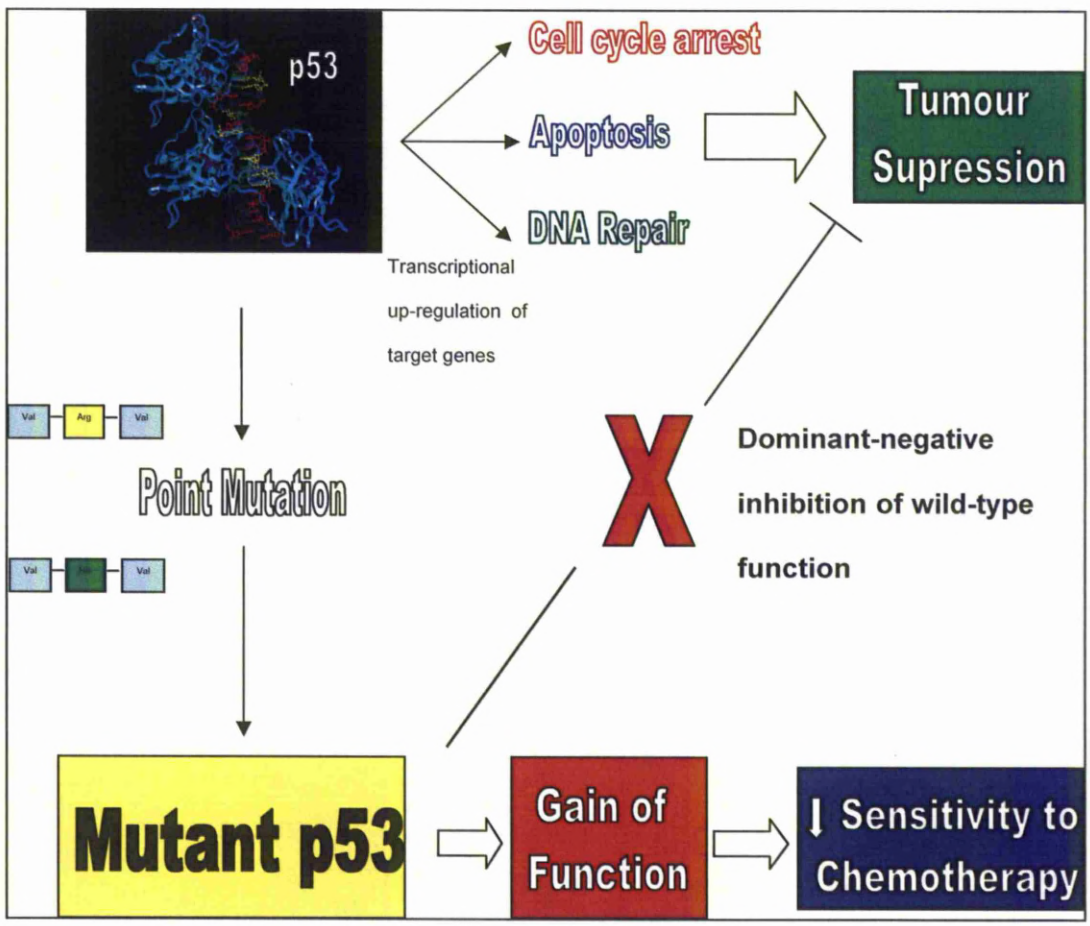



***Figure 1.14: Inhibiting mutant p53 in colorectal cancer cells may reduce malignant potential and augment response to adjuvant therapies.***

# Chapter 2. Materials and Methods

## 2.1 Buffers and Solutions

All buffers and solutions were prepared in water with resistance of $\geq 15$ MΩ.

All chemicals were obtained from Sigma Aldrich unless otherwise stated.

### 2.1.1 SLIP (Stuart Linn immuno-precipiation) Buffer (100ml)

50ml of 0.1M HEPES (pH 7.5)

10mls 10% (v/v) glycerol

0.1ml 0.1% (v/v) Triton X-100

150mM NaCl

0.05g of BSA.

Made freshly every seven days and stored at 4°C.

### 2.1.2 PBS (phosphate buffered saline) / Tween (4 L)

44.8g $Na_2HPO_4.2H_20$

9.67g $NaH_2PO_4.2H_20$

17.52g NaCl

4ml 0.1% (v/v) Tween 20 (polyoxyethylene sorbiton monolaurate).

### 2.1.3 Tris–Glycine Electrophoresis Buffer (500ml / tank)

25mM Tris-HCl

250 mM glycine

0.1% SDS.

**2.1.4 Electrophoresis Transfer Buffer** (1L / tank)

25mM Tris-HCl

192 mM glycine

20% methanol.

**2.1.5 4X Sample Buffer**

0.25 M Tris-HCl (pH 6.8)

8% SDS

40% glycerol

4mg/ml bromophenol blue

1% β–mercaptoethanol

Diluted to 2X and 1X with $H_2O$ as required.

**2.1.6 SDS Polyacrylamide Stacking Gel** (10ml)

7.225ml $H_2O$

1.275ml 40% acrylamide mix

1.25ml 1 M Tris-HCl (pH 6.8)

0.1ml 10% SDS

0.1ml 10% APS

0.01ml TEMED.

### 2.1.7 SDS Polyacrylamide Seperating Gel (10ml)

*Table 2.1: SDS Polyacrylamide Separating Gel Formulations.*

*Reproduced from Harlow and Lane(1999) Using antibodies : a laboratory manual [253].*

| | % Gel Concentration | | | | |
|---|---|---|---|---|---|
| | **6%** | **7.5%** | **10%** | **12%** | **15%** |
| **$H_20$** | 5.8ml | 5.42ml | 4.8ml | 4.3ml | 3.55ml |
| **40% w/v acrylamide solution** | 1.5ml | 1.87ml | 2.5ml | 3ml | 3.75ml |
| **1.5 M Tris (pH 8.8)** | 2.5ml | 2.5ml | 2.5ml | 2.5ml | 2.5ml |
| **10% SDS** | 0.1ml | 0.1ml | 0.1ml | 0.1ml | 0.1ml |
| **10% APS** | 0.1ml | 0.1ml | 0.1ml | 0.1ml | 0.1ml |
| **TEMED** | 0.008ml | 0.008ml | 0.008ml | 0.008ml | 0.008ml |

### 2.1.1 Cryogenic preservation media

10% DMSO (dimethyl sulphoxide)

90% FBS.

### 2.1.2 Pepstatin (Roche Applied Science)

Stored as 1000X stock at -80°C

1mg/ml in 100% methanol.

### 2.1.3 Leupeptin (Roche Applied Science)

Stored as 1000X stock at -80°C

0.5mg/ml in $H_2O$.

### 2.1.4 Aprotinin (Roche Applied Science)

Stored as 1000X stock at -80°C

2mg/ml in PBS.

### 2.1.5 Soybean Trypsin inhibitor (Roche Applied Science)

Stored as 1000X stock at -80°C

100µg/ml in $H_2O$.

### 2.1.6 Phenyl methyl sulfonyl fluoride (PMSF) (Sigma)

Fresh 100X stock of 100mM in 100% ethanol prepared each time.

### 2.1.7 Ponceau S (Sigma) 10X stock

2% Ponceau S (3-hydroxy-4-[2-sulpho-4-(sulpho-phenylazo)phenylazo]-2,7 napthalene disulphonic acid)

30% trichloroacetic acid

30% sulphosalicylic acid.

### 2.1.8 Annealing Buffer

100mM NaCl

50mM HEPES – pH 7.4.

### 2.1.9 Agarose Gel

1% gel = 1gram of Seakem GTG agarose (Cambrex biosciences Rockland inc.)

Dissolved in 100ml T.A.E. (Tris, Acetic Acid, EDTA).

0.5 µg/ml ethidium bromide added to all agarose gels (Roche)

### 2.1.10 Luria-Bertani Media (LB media aka LB -broth) (500ml)

12.5 grams powdered L.B. (Sigma) made to 500ml with water.

### 2.1.11 Luria-Bertani Agar (LB Agar) (500ml)

12.5 grams powdered L-Broth

7.5 grams Difco-bacto (or Oxoid) agar – made up to 500ml with water.

### 2.1.12 Orange G (Sigma)

Reconstituted in 10% glycerol with orange G powder added until appropriate orange colouration is achieved.

### 2.1.13 β-gal Fixing Buffer

0.5% glutaraldhyde in phosphate buffered saline.

### 2.1.14 β-Gal Substrate Buffer

3mM potassion ferrocyanide

3mM potassium ferricyanide

1mM $MgCl_2$

0.5mg/ml X-gal (5-bromo-3-indoyl-β-D-galactopyranoside)

Diluted in PBS.

## 2.2 Cell lines

### 2.2.1 Colorectal Cell lines

Unless otherwise stated, cell lines were grown from established frozen stocks obtained for our laboratory.

#### *2.2.1.1 HCT 116 and derivatives*

This tumour cell line was derived from a male patient with colon cancer.

The p53 status is documented as wild-type.

The heterozygous +/- and the homozygous -/- cells contain a ΔN40 mutation, on one or both alleles respectively.

This cell line and its derivatives were kindly donated by Dr Lorna Warnock from Prof. Joe Milner's group at the Yorkshire Cancer Research Laboratory at the University of York.

#### *2.2.1.2 LoVo*

LoVo was initiated in 1971 from a fragment of a metastatic tumour nodule in the left supra-clavicular region of a 56-year-old Caucasian male patient with a histological diagnosis of adenocarcinoma of the colon.

The original tumour was Duke's C, Grade IV.

The p53 status is documented as wild-type.

#### *2.2.1.3 SW480*

This transformed cell line was established from a Duke's B primary adenocarcinoma of the colon in a 50 year old Caucasian male in 1978. The p53 status is mutant with the cells expressing elevated levels of p53 protein. There is a G to A mutation at nucleotide 14487, exon-8, codon 273, which leads to a CAT mutant codon. This results in an Arginine to Histidine amino acid substitution and consequently a missense mutation (R273H). There is also a C to T mutation at nucleotide 14686, exon-9, codon 309, which results in a TCC mutant codon. This leads to a Proline to Serine missense substitution (P309S) [173]. The SW480 cell line has been demonstrated, by karyotyping and sequencing, to have three copies of chromosome 17 with all three copies of the gene containing the same two missense point mutations.

#### *2.2.1.4 SW620*

This cell line was cultured from the metastatic lymph node of the same patient as SW480, 1 year after the initial colonic resection. A recurrence of the malignancy resulted in a wide spread metastasis from the colon to an abdominal mass. The established cell line consists mainly of individual, small spherical and bipolar cells lacking microvilli. Levels of p53 protein expression are elevated. There is the same G to A mutation at codon 273 as found in the SW480 cell line. Interestingly, although this cell line is established from a later metastasis, it only has one p53 mutation whereas the tumour specimen cell line established on year previously carries two missense mutations [173].

#### *2.2.1.5 HT29*

This cell line was established from the colon cancer of a 44 year old female caucasian patient in 1964. The p53 gene is mutant with the same 273 Arg. to His (R273H) missense mutation in exon 8, as described in the SW480 and SW620 cell lines.

### 2.2.2 Other tumour cell lines

#### *2.2.2.1 PANC 1*

This cell line was established from a ductal pancreatic carcinoma in a 56 year old caucasian male. The cells have high levels of mutant p53 protein expression. They contain the same common R273H mutation, as the SW480, SW620 and HT-29 lines described above. They also have an additional mutation at codon 272 which is a T to C point mutation resulting in an alanine substitution.

#### *2.2.2.2 H1299*

A non small lung cancer cell line derived from a patient a metastatic lymph node.
The p53 status of this line is null.

#### *2.2.2.3 MCF-7*

A human breast adenocarcinoma cell line derived from a pleural effusion.
The p53 status of this line is wild-type.

## 2.3 Tissue Culture

All tissue culture was carried out using aseptic technique and performed in a class II laminar flow tissue culture cabinet. Cells were cultured as a monolayer at 37°C with 5% $CO_2$. They were routinely maintained in 175cm$^2$ tissue culture flasks and split every 48–72 hours, when at a confluence of 70-90%. Cells were cultured in either Dulbecco's Modified Eagles' Medium or RPMI – 1640, supplemented with 10% Foetal Bovine Serum and L-glutamine (all supplied by Sigma). All media, PBS and trypsin were warmed to 37°C prior to use.

***Table 2.2: Cell culture Methodology for various cell lines***

| Cell Line | Media | Routine Split Ratio | Trypsinization Time |
|---|---|---|---|
| **HCT 116 (and derivatives)** | DMEM -10% FBS 2mM L-Glutamine | 1:8 | 2 minutes |
| **LoVo** | DMEM -10% FBS 2mM L-Glutamine | 1:5 | 3 minutes |
| **SW480** | DMEM -10% FBS 2mM L-Glutamine | 1:8 | 1 minute |
| **SW620** | DMEM -10% FBS 2mM L-Glutamine | 1:8 | 1 minute |
| **HT29** | DMEM -10% FBS 2mM L-Glutamine | 1:5 | 5 minutes |
| **H1299** | RPMI – 10% FBS | 1:10 | 1 minute |
| **Panc 1** | RPMI – 10%FBS 2mM L-Glutamine | 1:5 | 2 minutes |
| **MCF-7** | RPMI – 10%FBS 2mM L-Glutamine | 1:8 | 1 minute |

### 2.3.1 Cell harvesting and passage

Culture media was removed and cells washed with sterile Dulbecco's PBS (Sigma) to remove any residual media. A 0.25% Trypsin – 0.53mM EDTA solution was applied in a thin layer and cells incubated as above until they were no longer adherent to the flask base, as confirmed by light microscopy. The trypsin was neutralised with an equal or greater volume of the appropriate media containing FBS. The cells were repeatedly aspirated and released from a glass pipette to produce a single cell suspension and then re-plated at a density appropriate for each cell line.

### 2.3.2 Freezing and thawing cells

Cells were harvested with trypsin as per 2.3.1. Cells were spun in a centrifuge at 300rcf, 4°C for 5 minutes. The pellet was then re-suspended in freezing media and the cryo-vial kept at -80°C for 24 hours prior to storage in liquid nitrogen at a concentration of $1\text{-}5x10^6$/ml. Cells were defrosted at 37°C and immediately transferred into pre-warmed culture media.

## 2.4 Western Blotting

### 2.4.1 Cell lysate preparation

Cell pellets were lysed on ice with SLIP buffer (2.1.1) containing the protease inhibitors pepstatin, leupeptin, aprotinin, soybean trypsin inhibitor and PMSF (2.1.9 - 2.1.13). SLIP buffer was added to each pellet until the dissolved solution was just pearlescent and left on ice for 10 minutes. Samples were then spun in the centrifuge at 14,000 x rcf at 4°C for 10 minutes to remove any cellular debris and the consequent supernatant was then aspirated and retained and the pellet discarded.

### 2.4.2 Bradford assay to determine protein concentrations

A calibration curve of standard protein concentrations was created using bovine serum albumin (Sigma) made up in SLIP (with protease inhibitors as above) to concentrations of 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0 mg/ml. Bradford protein assay reagent (Biorad) was diluted 1:5 with water. 2µl of each standard was added to 1ml of the Bradford reagent and the optical density of each sample at 595nm, measured using a spectrophotometer. Discrepancies of <5 in the c.v. reading were deemed acceptable. Protein concentrations of the supernatants were determined in a similar fashion and then adjusted with 4X, 2X and 1X sample buffer (2.1.5) to a final concentration of 50µg/20µl for the majority of samples. Occasionally, when protein yields were lower, lysates were prepared at 30µg/20µl; this is indicated where appropriate. Samples were either stored at -80°C until required or prepared immediately for resolution on an SDS Page Gel. Prior to loading, lysates were boiled for five minutes to denature the proteins and then spun at 14,000 x rcf, at 4°C for 4 minutes before returning onto ice immediately prior to loading.

### 2.4.3 SDS-Page

0.75mm glass plates were cleaned with 70% ethanol, air dried and mounted. An SDS polyacrylamide separating gel (2.1.7) was poured between the plates to within 1.5cm of the top. The percentage of gel used was dependant on the apparent molecular weight of the proteins to be probed and therefore was experiment dependant. The gel was covered with $H_2O$ and left to set for 20 minutes before removing the water and adding the stacking gel (2.1.6). A 10 well comb was placed into the stacking gel and allowed to set for 15 minutes before removal of the comb and washing out of the wells with water. The gel and plates were then transferred to a Mini Protean III electrophoresis chamber (Biorad) and Tris-glycine electrophoresis buffer (2.1.3) added to fill the central chamber and half the external chamber (500ml / chamber). 20µl of sample were loaded alongside 15µl of broad range pre-stained molecular weight marker (New England Biolabs) and subjected to electrophoresis at 200V for 55-70 minutes. The gel apparatus was dismantled, the stacking gel removed and a Hybond ECL nitrocellulose membrane (Amersham Biosciences) placed on the gel and sandwiched between 3MM Whatman chromatography paper (VWR) and sponges. This procedure was performed submerged in transfer buffer (2.1.4) to prevent air bubbles from forming within the 'sandwich'. The nitrocellulose membrane was pre-soaked in the transfer buffer to ensure adequate hydration. The assembled transfer apparatus was then returned to the chamber, along with an ice block for cooling, the chamber filled with the buffer (2.1.4) and transferred for 1 hour at 100V. The membrane was then removed, stained in Ponceau S (2.1.14) for 1 minute, washed in water and photographed to assess evenness of loading and efficiency of transfer. It was cut longitudinally, at levels dictated by the apparent molecular weights of the proteins to be studied and using the pre-stained marker as a

guide. The membrane underwent several washes in PBS / Tween (2.1.2) before being incubated overnight in 5% blotting grade non-fat dry milk powder (Biorad) in PBS / Tween, to block any non-specific protein binding sites.

### 2.4.4 Immunoblotting

The membrane was agitated at room temperature for one hour. Primary antibodies were diluted with 5% milk powder in PBS/ Tween, with concentrations and suppliers as per Table 2.3. Incubation with the primary and consequently the secondary antibodies, was for 1 hour each, at room temperature, with continuing agitation. The blot was washed in PBS / Tween for three cycles of ten minutes after each antibody had been removed.

***Table 2.3: Primary antibodies used in immuno (ie western)-blotting***

| Antibody | Clone or Variant (product code) | Supplier | Final concentration | Source |
|---|---|---|---|---|
| p53 | DO-1 (Ab 6) | Oncogene/Merck | 0.02– 3µg/ml | Mouse mAb |
| p53 | Ab 2433 | Abcam | 1:2,000 | Rabbit pAb |
| Actin | C2 | Insight | 3µg/ml | Mouse mAb |
| MDM2 | (IF-2) Ab 1 | Oncogene/Merck | 3µg/ml | Mouse mAb |
| MDMX | D19 | Santa Cruz Biotechnology | 1:5,000 | Goat pAb |
| Pirh2 | pAb BL588 | Bethy Labs. | 1:1,000 | Rabbit pAb |
| Cdkm1a | F5<br>sc-6246 | Calbiochem | 3µg/ml | Mouse mAb |
| p14ARF | C18 | Calbiochem | 1:500 | Goat pAb |
| MTBP | AS–1 | Manufactured from in house peptide raised against human MTBP | 1:1,000 | Rabbit pAb |
| β-Gal | Ab-1 | Oncogene/Merck | 3µg/ml | Mouse mAb |
| GFP | Clones 7.1 and 13.1 | Roche Applied Science | 0.4µg/ml | Mouse mAb |

Secondary antibodies.

Anti–mouse IgG, horse radish peroxidase (HRP)-linked whole antibody, Amersham (1mg/ml used at [1:2,500] final concentration)

Anti-rabbit IgG, HRP linked whole antibody, Amersham (1mg/ml used at [1:5000] final concentration).

Rabbit, anti-goat IgG, HRP conjugate, Zymed

(1.5mg/ml used at [1:5000] final concentration)

### 2.4.5 Developing

Western blots were visualised using enhanced chemo-luminescence (ECL) reagent plus (GE Healthcare). Equal quantities of the enhanced luminal and oxidising reagents were combined and applied to cover the entire blot for one minute. The membrane was then blotted dry, reassembled, wrapped in cling film and secured in a Kodak light safe developing cassette. Blots were exposed to Fuji medical x-ray film at a range of time periods to optimise exposures for detection of the various proteins being studied. The film was subsequently developed in Kodak developer and fixer for two minutes respectively and then rinsed in water and allowed to air dry.

## 2.5 Luciferase reporter assay

In order to study p53 regulated transcriptional activity, a DNA plasmid luciferase reporter system was utilised. The pp53-TA-Luc vector (Clontech laboratories inc.) contains a p53 response element upstream of a herpes simplex virus thymidine kinase promoter ($P_{TA}$). Located downstream of $P_{TA}$ is the firefly luciferase reporter

gene, hence luminescence can be equated to levels of p53 mediated signal transduction.

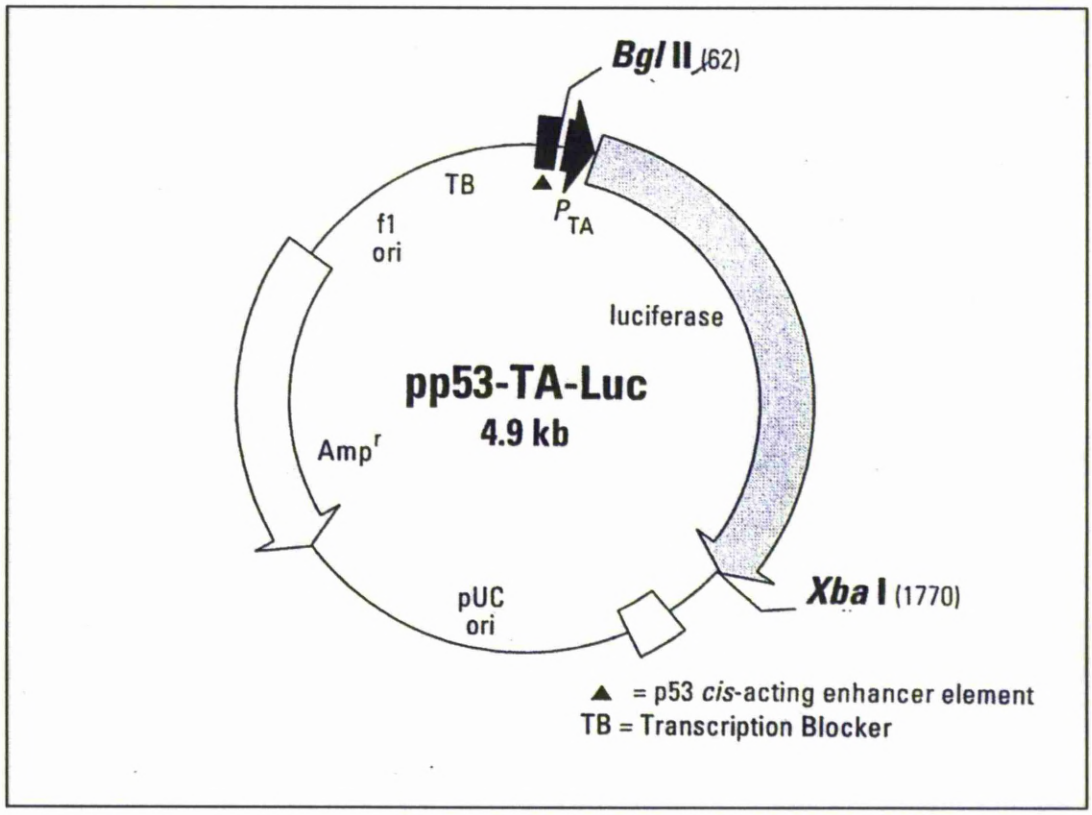



*Figure 2.1: The pp53-TA-Luc Luciferase Reporter construct.*

*Pubished by Clontech laboratories Inc. pp53-TA-Luc Vector Information, Protocol No. PT3511-5W2. Version No. PR19431. www.clontech.com*

## 2.6 SiRNA knock down experiments

### 2.6.1 SiRNA Oligonucleotides

The following siRNA molecules were used in this study, and were obtained from Dharmacon RNA technologies.

**MDM2 (#1)**

Sense sequence - 5' GCCACAAAUCUGAUAGUAUUU 3'

Anitisense sequence - 5' AUACUAUCAGAUUUGUGGCUU 3'

**Dharmacon C1 control**

Sense Sequence - 5' UAGCGACUAAACACAUCAAUU 3'

Antisense Sequence - 5' UUGAUGUGUUUAGUCGCUAUU 3'

**Wild Type p53 (Martinez 2002 [244])**

Sense sequence - 5' GCAUGAACCGGAGGCCCAUUU 3'

Antisense sequence - 5' AUGGGCCUCCGGUUCAUGCUU 3'

The following siRNAs were designed by M Boyd using the online engine at the Whitehead Institute, http://sirna.wi.mit.edu/

**p53 R273H mutant #1**

Sense sequence - 5' AGCUUUGAGGUGCAUGUUUUU 3'

Antisense sequence - 5' AAACAUGCACCUCAAAGCUUU 3'

**p53 R273H mutant #2**

Sense sequence - 5' CUUUGAGGUGCAUGUUUGUUU 3'

Antisense sequence- 5' ACAAACAUGCACCUCAAAGUU 3'

**p53 R273H mutant #3**

Sense sequence - 5' ACAGCUUUGAGGUGCAUGUUU 3'

Anitsense sequence - 5' ACAUGCACCUCAAAGCUGUUU 3'

**Scrambled ('MTBP' siRNA sequence)** [117]

Sense Sequence - 5' GGACGCAUCCUUCUUAAUUUU 3'

Antisense sequence - 5' AAUUAAGAAGGAUGCGUCCUU 3'

### 2.6.2 SiRNA: Methodology of basic knock-down experiments

These experiments were carried out in 6 well tissue culture plates (each well with an area of 9.5cm$^2$), with each condition / treatment being applied to the 6 wells of one plate. Cells were harvested with trypsin from their routine culture flasks and seeded, in their usual media, to aim for a confluence of 40% at the start of transfection, the following day.

Approximately 24 hours post plating, cells were washed with sterile PBS and then 2mls of fresh media were applied per well. The cells were then transfected with the luciferase reporter plasmid and a β-galactoside expressing plasmid. The β-galactoside plasmid is driven by a Simian Virus (SV40) Promoter and is utilised as a marker for transfection efficiency under the assumption that expression of this should be unaffected by the experimental conditions. It is hypothesised that the levels of the luciferase reporter plasmid incorporated into the cells will be proportional to the β galactoside levels detectable by western-blotting.

Approximately 1.18μg of pp53-TA-Luc plasmid / well was combined with 0.42μg of B-Gal plasmid / well and mixed thoroughly. The transfection reagent GeneJuice

(Novagen), was used at a volume of 3μl per μg of DNA being transfected (i.e. 4.2μl per well). This was combined with 100μl of serum free media (the same media used for the cells normal tissue culture without the FBS added) / well, mixed thoroughly and left at room temperature for five minutes. The two solutions were combined, mixed gently and left for fifteen minutes at room temperature. 100μl of this solution was added to each well and the cells incubated for 4 hours before being replaced with 1.6ml of fresh media.

The siRNA oligonucleotides were used at a final concentration of [40nM] except in the optimisation experiments (see 4.2). A scrambled siRNA sequence (produced from a rearranged MTBP siRNA) and a commercially purchased control sequence (Daramacon) C1 were used as negative controls, designed to be non-targeting against the cellular DNA and hence helping to confirm the specificity of any knock down effects. A lipofectamine only treatment group was also studied to account for transfection reagent mediated toxicity. The siRNA was added to Opti-mem media (invitrogen) at a volume of 200μl / well and a further 200μl of opti-mem was combined with 4μl /well of the transfection reagent Lipofectamine 2000 (Invitrogen). Both of these solutions were mixed thoroughly and left for 5 minutes before being combined, mixed gently and left for a further twenty minutes. 400μl was then added to each well and the cells incubated for a further six hours. At this stage the old original media was removed and fresh applied to negate the possible toxic effects of prolonged exposure to the Lipofectamine reagent. Typically, forty-eight hours after the application of the siRNA solution the cells were harvested (excluding optimisation experiments – see 4.2). Three wells from each condition were harvested using Trypsin-EDTA and spun in a centrifuge and the supernatant was subsequently

removed by aspiration to produce a pellet for western blot analysis as described in section 2.4.

The remaining 3 wells were treated as separate samples for the Luciferase Reporter Assay (see 2.4). A Glo-lysis luciferase reporter assay kit (Promega) was used. To perform the assay, media was removed by aspiration and the cells were then washed twice with sterile PBS to remove traces of media which could interfere with the assay. The cells were then lysed with a specific volume of the Glo-lysis buffer at room temperature and agitated on a shaking table for 10 minutes. The volume of glo-lysis buffer necessary to lyse the cells was constant within each individual experiment, but varied from 300 – 500 µl, as necessary, to adequately lyse the specific cell line in use. Cells were harvested and transferred into 1.5ml micro-centrifuge tubes (Eppendorf), mixed with a pipette and centrifuged at 16,100 x rcf in a desk-top centrifuge for 3 minutes to pellet cellular debris. The supernatant was decanted and 50µl of this was added to 50µl of the luciferase reagent (at room temperature). This was immediately placed in a Turner Design 20/20 Luminometer and readings were taken for a thirty second period following an eight second pre-reading pause. Results were recorded in relative light units (RLU). The samples were normalised to RLU / µg protein using a Bradford assay to estimate protein concentrations (2.4.2). The mean of the three normalised results was plotted with the standard error of the mean (SEM) used and presented as the Y-axis error bars.

## 2.7 In situ β Gal assay

Cells were plated the day before transfection into one 10cm dish per cell line, aiming for ca. 40% confluence on the day of transfection. β-galactoside and pp53-TA-Luc vectors were transfected as outlined previously in section 2.6.2 . Twenty-four hours later the media was removed, the cells washed with sterile PBS twice and 10ml of fixing buffer (2.1.20) added per dish and left at room temperature for 15 minutes. This was removed and the cells again washed with PBS, prior to the application of 5-10ml per dish of substrate buffer (2.1.21). The cells were incubated at 37°C for 3 – 8 hours, after which the substrate buffer was removed and the plates washed twice with water. Once dried, the plates were visualised by microscopy to assess the number of positively transfected (blue) cells and a photographic record made.

## 2.8 pSUPER plasmid preparation and cloning

### 2.8.1 The pSUPER vector

The pSUPER RNAi system (OligoEngine) is a commercially available DNA plasmid that directs intracellular synthesis of siRNA-like transcripts. The pSUPER vector has been shown to cause efficient and specific down regulation of gene expression, resulting in functional inactivation of the targeted genes. Stable expression of siRNAs using this vector mediates persistent suppression of gene expression, allowing the analysis of phenotypes that develop over longer periods of time.

The pSUPER vector is used with a pair of custom oligonucleotides that contain a 19-nuleotide sequence derived from the mRNA transcript of the gene targeted for suppression. The 19 nucleotide target sequence corresponds to the sense strand of the

pSUPER-generated siRNA. In the mechanism of RNAi, the antisense strand of the siRNA duplex hybridises to this region of the mRNA to mediate cleavage of the molecule, as shown in Figure 2.2.

The designed oligonucleotides (see section 2.8.2) include the 19 base target sequence in sense and antisense orientation separated by a 9-nt spacer sequence that allows formation of a short hairpin (hence shRNA) when transcribed. The vector uses the polymerase-III-H1-RNA gene promoter, as it produces a small RNA transcript lacking a polyadenosine tail and has a well-defined start of transcription and a termination sequence consisting of five thymidines in a row. Cleavage of the transcript (by Dicer (see section 1.7.3) occurs at the termination site after the second uridine yielding a transcript resembling the ends of synthetic siRNAs, which also contain two 3' overhanging T or U nucleotides.

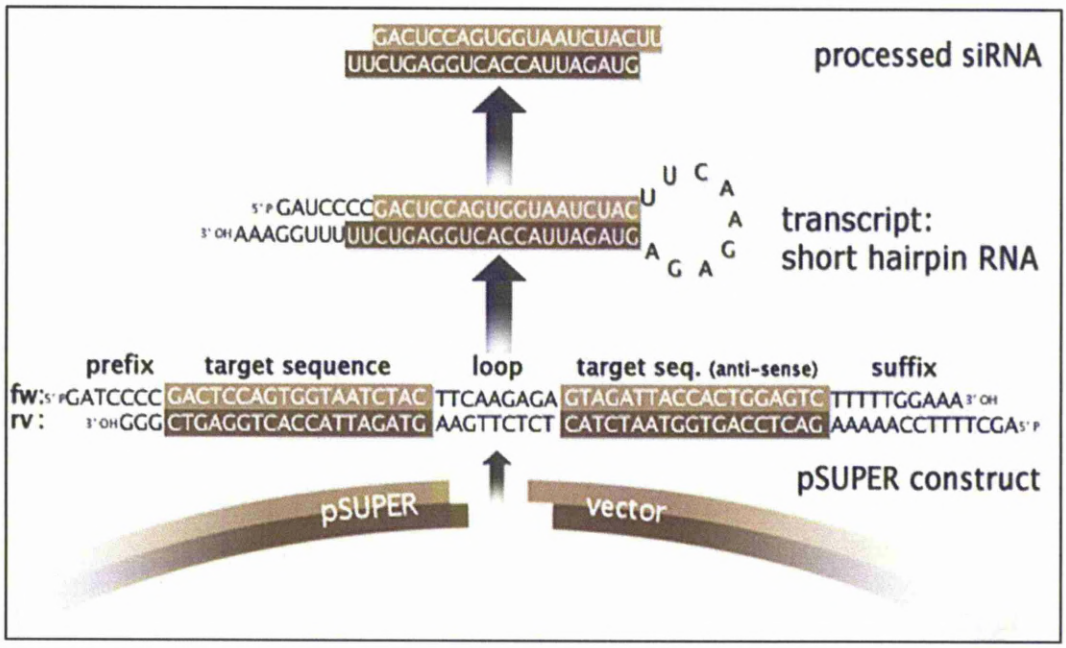



***Figure 2.2: Transcription of 60 base pair olignucleotide into hairpin RNA and processing to produce siRNA.***

***(Reproduced from pSUPER manual, www.oligoengine.com/products/psuper.html)***

The pSUPER vector backbone has a length of 5,429 base pairs. The vector contains a neomycin resistance gene allowing only stably transfected clones to survive under conditions of antibiotic selection, thus theoretically overcoming the problems associated with poor transfection efficiency observed with some cell lines. The pSUPER vector also has an ampicillin resistance gene which is utilised in as a bacterial selection marker during transformation. In addition, the plasmid contains an Enhanced Green Fluorescence Protein (eGFP) gene, which is fused with the neomycin sequence. eGFP is a gene isolated from jelly fish that allows them to glow luminous green in response to an ultra-violet light stimulus. The fusion gene is driven from a phosphoglycerate kinase (PGK) promoter. The vector contains T3 and M13 reverse primers to allow sequence confirmation of the correct insert.

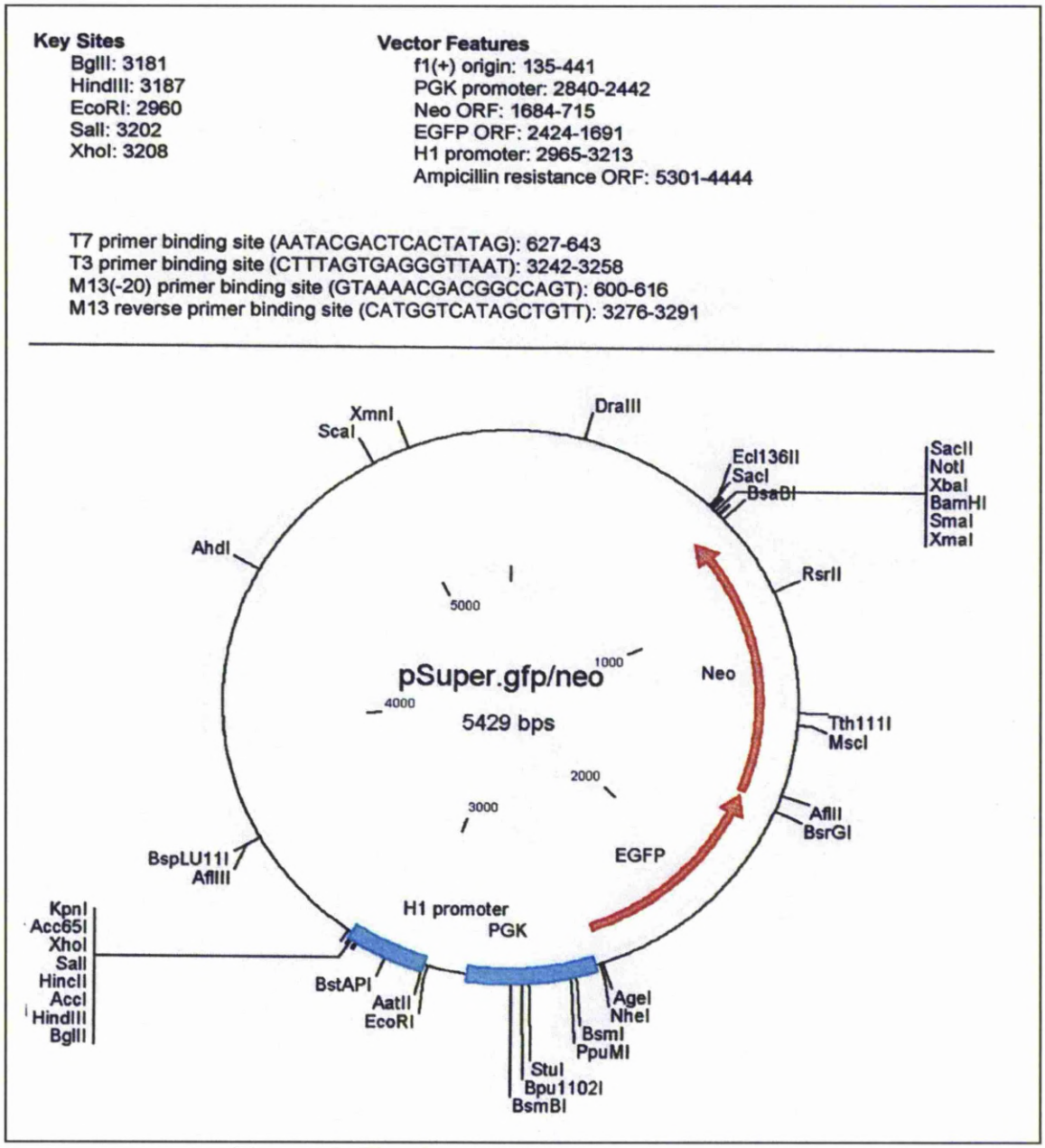



*Figure 2.3: The pSuper GFP / Neomycin Vector.*

*(Reproduced from pSUPER manual, www.oligoengine.com/products/psuper.html*

### 2.8.1 The oligonucleotide sequences

Antisense sequence in **RED**

Sense sequence in **BLUE**

**pSUPER scrambled Top:**

5' – GAT CCC C**GG ACG CAT CCT TCT TAA TT**T TCA AGA GA**A ATT AAG AAG GAT GCG TCC** TTT TAA - 3'

**pSUPER scrambled Bottom:**

5' – AGC TTA AAA A**GG ACG CAT CCT TCT TAA TT**T CTC TTG AA**A ATT AAG AAG GAT GCG TCC** GGG – 3'

**pSUPER wild-type p53 Top:**

5' – GAT CCC C**GC ATG AAC CGG AGG CCC AT**T TCA AGA GA**A TGG GCC TCC GGT TCA TGC** TTT TTA – 3'

**pSUPER wild-type p53 Bottom:**

5' – AGC TTA AAA A**GC ATG AAC CGG AGG CCC AT**T CTC TTG AA**A TGG GCC TCC GGT TCA TGC** GGG -3'

**pSUPER p53 #3 Top:**

5' – GAT CCC C**AC AGC TTT GAG GTG CAT GT**T TCA AGA GA**A CAT GCA CCT CAA AGC TGT** TTT TTA – 3'

**pSUPER p53 #3 Bottom:**

5' – AGC TTA AAA A**AC AGC TTT GAG GTG CAT GT**T CTC TTG AA**A CAT GCA CCT CAA AGC TGT** GGG – 3'

### 2.8.2 Annealing the oligonucleotides

Oligonucleotides were dissolved in nuclease free $H_2O$ to a concentration of 3 mg/ml. 3µg (1µl) of forward and reverse oligonucleotides were added to 48µl of Annealing Buffer (2.1.15). This mixture was incubated at 90°C for 4 minutes, 70°C for ten minutes, and then allowed to cool gradually to room temperature over the next 40 minutes. Samples were used immediately in the ligation reaction, as outlined see section 2.8.5, or transferred to -20°C for storage.

### 2.8.3 Linearising the pSUPER vector

The pSUPER vector was linearised in sequential enzymatic digestions:

***Table 2.4: Linearising the pSUPER vector: Hind III and Bgl II Digests***

Stage 1 - Digestion with Hind III (New England Biolabs) for 60 minutes at 37°C

| | CUT | UNCUT |
|---|---|---|
| pSUPER Vector | 5µg (10µl) | 0.5µg (1µl) |
| Hind III enzyme (50 units) ( @ 20,000U/ml) | 2.5µl | N/A |
| Buffer No. 2 (New England Biolabs.) | 5µl | 5µl |
| $H_2O$ | 32.5µl | 44µl |

Stage 2 – Digestion with Bgl II (New England Biolabs.) for 120 minutes at 37°C

| | CUT | UNCUT |
|---|---|---|
| Bgl II (50 units) (@10,000U/ml) | 5µl | N/A |
| Buffer No. 3 (New England Biolabs.) | 5µl | 5µl |
| $H_2O$ | N/A | 5µl |

These reactions were heat inactivated at 80°C for 20 minutes.

The linearised product was run alongside the uncut vector on a 1% agarose gel (2.1.16) to confirm digestion. The band corresponding to the linear backbone was then excised from the gel and purified using a GENECLEAN Turbo Kit (Q BIOgene) according to the manufacturer's instructions. 3µl of the product of this was run again on a 1% agarose gel alongside a 1 Kb ladder (Invitrogen) to estimate the concentration of backbone DNA. Typically, 3µl of sample corresponded to approximately 100ng of DNA, therefore this was the quantity used in the ligation reaction.

### 2.8.4 Ligation into the pSUPER vector

The annealed oligonucleotides were used at ratios of 1:1, 1:10, and 1:100 of vector : Oligo with 100ng of vector per ligation reaction.

i.e. (ng vector x Kb insert) / Kb vector x molar ratio = ng insert.

( 100 x 0.06 ) / 5.4 x 1 = 1.11 ng (1:1)
( 100 x 0.06 ) / 5.4 x 10 = 11.11 ng (10:1)
( 100 x 0.06 ) / 5.4 x 100 = 111.11 ng (100:1)

The ligation reactions were run as shown in Table 2.5 and incubated overnight at room temperature.

***Table 2.5: Annealed oligonucleotide ratios for ligation reaction into the pSUPER vector***

| | 1:1 | 1:10 | 1:100 | No insert control |
|---|---|---|---|---|
| Backbone | 3µl/100ng | 3µl/100ng | 3µl/100ng | 3µl/100ng |
| Oligo | 0.009ml (1.11ng) | 0.09ml (11.11ng) | 0.9ml (111.11ng) | N/A |
| T4 DNA Ligase (Ambion) | 1µl | 1µl | 1µl | 1µl |
| T4 DNA Ligase Buffer (Ambion) | 1µl | 1µl | 1µl | 1µl |
| $H_20$ | 4.991µl | 4.91µl | 4.1µl | 5µl |

The Bgl II site in the pSUPER backbone is destroyed upon successful insertion of the oligonucleotide sequence. Therefore, after cloning and prior to transformation, the plasmids were incubated with Bgl II 10 units/10µl reaction) for 30 minutes at 37°C, to reduce the level of background in the transformation.

### 2.8.5 Bacterial Transformation

L.B. agar (2.1.18) was melted and cooled until just liquid. Ampicillin [100µg/ml] was added and the mixture was then poured into 10cm bacterial plates and allowed to set. 5µl of each ligation reaction was added to 50µl of One Shot Top 10 chemically

compotent E. coli (Invitrogen) and incubated on ice for thirty minutes. The cells were heat-shocked for 30 seconds at 42°C and returned to ice. 250µl of SOC media (Invitrogen) was added and incubated at 37°C, with agitation at 225 rpm, for one hour. Various amounts of the cultures were then plated (typically 90% (270µl) and 10% (30µl)), and evenly spread across the plates before incubating overnight at 37°C. pUC-19 plasmid (Invitrogen) was utilised as a marker of transformation efficiency. 29 colonies were obtained from 1.66 x $10^{-7}$µg DNA, resulting in a transformation efficiency of 1.74 x $10^{8}$ colonies / µg DNA.

The vector to oligo ratio of 1:10 yielded the most colonies, the 1:1 was the next highest, and there were relatively few colonies from the 1:100 ratio. In all cases the 10% plate was utilised for subsequent analysis as the colonies were too confluent on the 90% plating. Eight colonies from the 10% plates of the 1:1 and 1:10 ligations respectively were selected for screening for each oligonucleotide insert. The colonies were inoculated aseptically into 3ml L.B. (2.1.17) with ampicillin [100µg/ml] and then incubated at 37°C overnight with agitation at 225rpm.

Plasmid DNA was purified from 2.5ml of each overnight culture using a QIAprep Miniprep Purification Kit (Qiagen) and then subjected to an EcoRI / Hind III double digest as described below, for two hours at 37°C, to identify clones containing inserts.

*Table 2.6: EcoRI / Hind III Digest to screen pSUPER clones*

| | CUT | UNCUT |
|---|---|---|
| DNA | 2µl (~1µg) | 2µl (~1µg) |
| ECO R1 (20,000U/ml) (New England Biolabs) | 0.5µl | N/A |
| Hind III (20,000U/ml) (New England Biolabs) | 0.5µl | N/A |
| ECO R1 Buffer (New England Biolabs) | 2µl | 2µl |
| $H_2O$ | 15µl | 16µl |

Following restriction enzyme digestion samples were combined with 3µl Orange G (2.1.19), and analysed on a 2% agarose gel (2.1.16), by electrophoresis at 80V for 2hours. The size of samples bands was estimated by comparison with a 100 base pair ladder (MBI Fermentas), as shown in Figure 2.4.

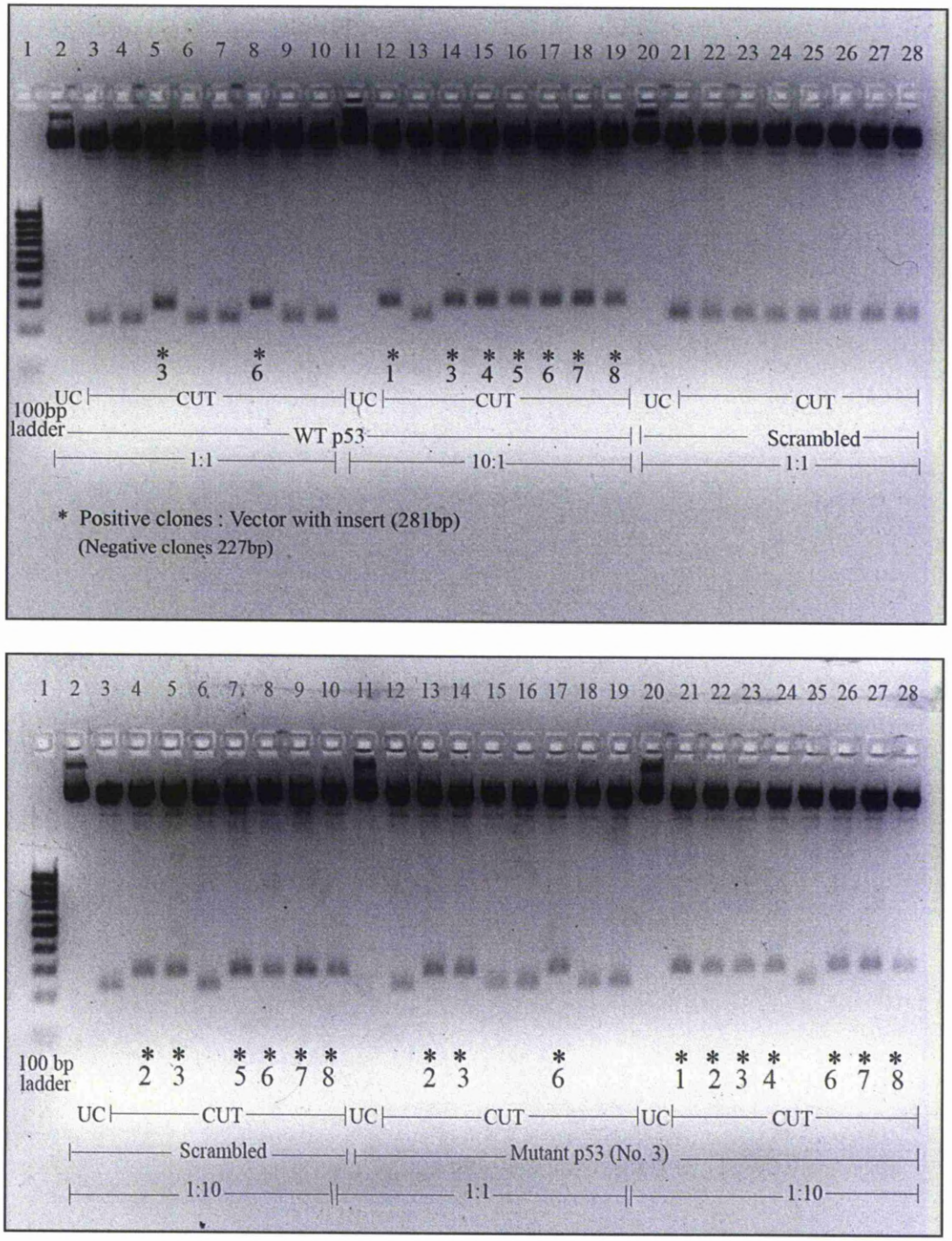



***Figure 2.4: Agarose Gel confirming multiple clones with positive inserts for each oligonucleotide sequence.***

*UC = Uncut (control samples with no Digestion enzymes)*

*C = Cut (The products of the EcoRI / Hind III digest)*

Frozen glycerol stocks were kept of all positive clones. For this 50% glycerol (Sigma) was sterilised by passage through a 0.2μm filter and then 500μl of this solution combined with 500μl of the overnight bacterial culture from which plasmid DNA was isolated prior to storage at -80°C.

Clones WT 1:1 No. 3, Scram 1:10 No. 2 and p53#3 1:1 No. 2 were selected to be amplified for transfection studies. Where possible 1:1 clones were used as these provided the lowest possibility of multiple inserts of the oligonucleotide sequence. To generate larger quantities of plasmids to be used for transfection experiments 500μl of selected overnight cultures was added to 500ml of L.B. (2.1.17) with ampicillin [100μg/ml] and incubated overnight at 37°C with 225rpm agitation. The plasmid DNA was then purified using the EndoFree Mega purification kit (Qiagen) according to the manufacturer's instructions. A further EcoRI / Hind III double digest was performed to confirm the presence of the successful oligonucleotide sequence insertion and the resultant samples were analysed by electrophoresis on a 2% agarose gel. As previously shown in Figure 2.4, the shRNA containing vectors produce a digest product of 281 base pairs compared with the 227 base pair parent vector, confirming successful insertion, as demonstrated for the large scale preparations in Figure 2.5.

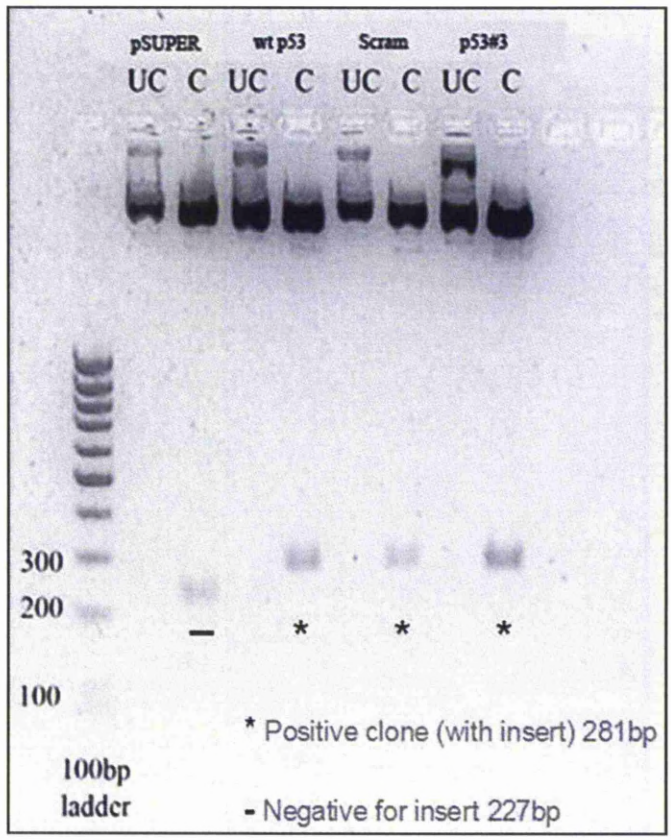



***Figure 2.5: Agarose gel analysis of large scale preparations of plasmid DNA taken from selected bacterial clones confirming the correct insertion of the Wtp53, mutant p53 and scrambled sequences.***

UC = Uncut (control samples with no Digestion enzymes)

C = Cut (The products of the EcoRI / Hind III digest)

Following identification of correctly cloned inserts by restriction analysis, plasmids were sequenced to confirm that the shRNA sequence was inserted correctly. Sequencing was performed by MWG Eurofins.

### *2.8.6* Antibiotic Dose Selection

Cell lines respond differently to the same dose of antibiotics, therefore a range of concentrations of G418 (neomycin) were tested, to determine which provided the most appropriate balance between death and viability. For example, if we use a very

high dose then all of the sensitive cells will be killed quickly. However, if the concentration is too high, cells that express the resistance gene, may not be able to proliferate. Thus a balance is required and for example using G418, in practise, this usually amounts to using a dose that kills the parental cells over a period of 5-10 days. G418 Sulphate (Calbiochem) was dissolved at a concentration of 50mg/ml in 0.1M HEPES, pH 7.3 and filter sterilised through a 0.2μm filter prior to use. Cells were plated into 6cm dishes, using their usual growing media, 24 hours prior to the first dose of antibiotics. Initial confluences of 20- 30% were recorded on Day 0 and the first dose of G418 was diluted in the appropriate media for each cell line and applied to the plates. Media was removed and antibiotic solutions replaced every 72 hours, with the percentage of confluent, viable cells being documented on a daily basis. The concentration used in the subsequent transfection studies is identified by the red line shown in Figure 2.6.

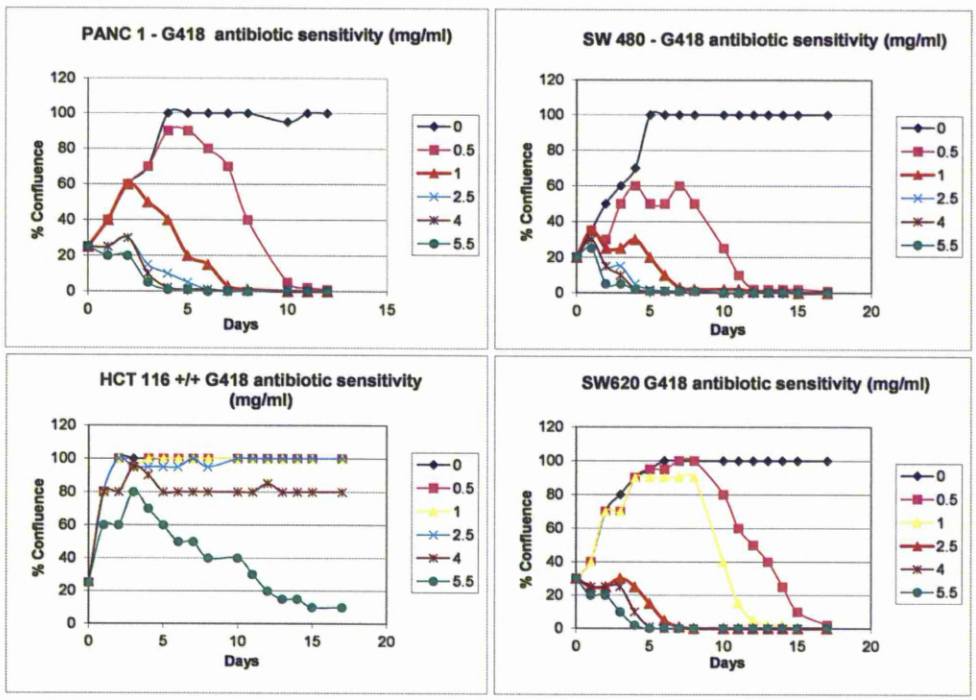



*Figure 2.6: G418 antibiotic sensitivity results*

HCT 116 +/+ cells continued to grow, even in the presence of high doses of G418, with viable cells still present following seventeen days of antibiotic treatment. Attempts were made to plate at lower initial confluences to hasten death of the cells, however, as these cells tend to grow in islands, there was a limit to how thinly they could be successfully plated. Therefore, it was decided to perform a puromycin kill curve, as the pSUPER vector is also available with a puromycin resistance gene for antibiotic selection. Puromycin (invitrogen) stock at 10mg/ml was diluted with tissue culture medium to the working concentrations in Figure 2.7.

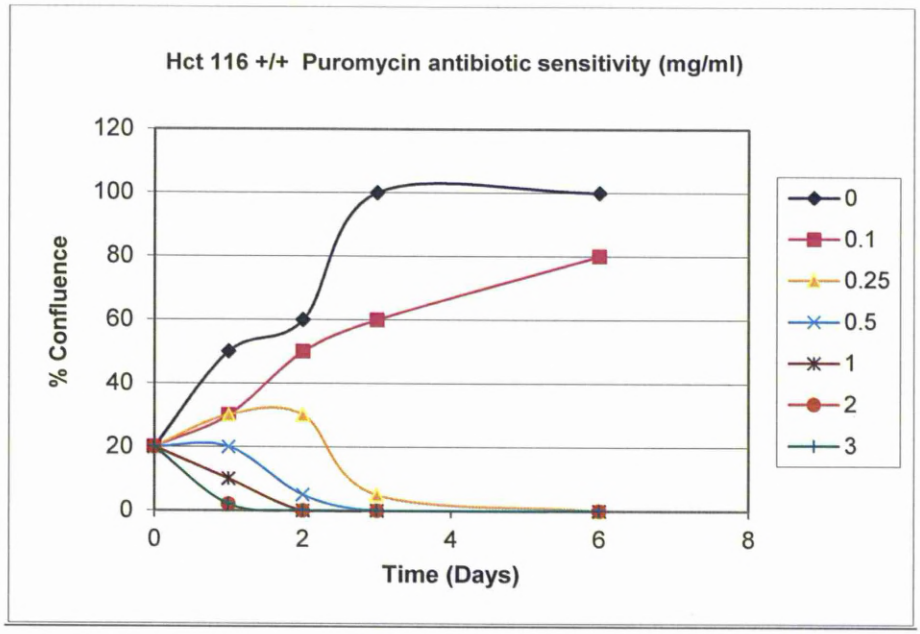



***Figure 2.7: Puromycin antibiotic sensitivity results for HCT 116 +/+ cell line.***

Figure 2.7 suggests a concentration of 0.2µg/ml would provide an appropriate selection for these cells. Ideally, 5 – 7 days to kill all cells is optimal and the doses 0.1 and 0.25µg/ml are either side of this timescale). However, cloning of shRNA oligonucleotides into the puromycin pSUPER vector was not undertaken within the scope of this thesis due to time constraints.

### 2.8.7 Transfection of p-SUPER vector into human tumour cell lines

Each cell line was transfected with pSUPER backbone alone and the pSUPER backbone with a scrambled sequence inserted (designed to be non-targeting against the cellular DNA and hence helping to confirm the specificity of any knock down effects). As well as the shRNA sequences designed to suppress both mutant and wild type p53. Cells were plated the day prior to transfection, in three wells of a six well plate per condition, to aim for confluence of 90% on the following day. Prior to transfection, the media was removed, the cells washed twice with sterile PBS and 1.5ml of fresh media added. 4μl of Lipofectamine 2000 (Invitrogen) was combined with 250μl Opti-mem (Invitogen) per well, vortexed and stored/retained/incubated at room temperature for five minutes. Similarly 2μg of the appropriate pSUPER vector was combined with a further 250μl of Opti-mem mixed thoroughly and stored/retained/incubated for five minutes. The plasmid and Lipofectamine solutions were combined, gently mixed and maintained at room temperature for twenty minutes, before applying 500μl per well. Twenty four hours later the cells were harvested with trypsin and re-seeded into three 6cm dishes per condition, aiming for confluences of 20-30% at the application of the first antibiotic dose as determined in Figure 2.6. Two non-transfected controls were also plated. The first was subjected to antibiotic selection to control for any spontaneous or inherent antibiotic resistant and to determine the effectiveness of the selection. The second control was grown without any antibiotic selection as a positive control for cell viability.

Media and antibiotics were changed every seventy two hours. Cells were grown until all of the cells in the untransfected antibiotic selection plates were dead and the

growing colonies were big enough to be seen easily with the naked eye. In the case of the SW480 cells, the initial dose of G418 suggested from the antibiotic selection test was 1mg/ml. However, when this dose was used, the untreated cells remained viable even after twenty days under selection and continued to multiply despite the antibiotic; therefore the selection dose was increased to 2mg/ml and the experiment repeated.

### 2.8.8 Selection of stable transfectants

To select individual colonies, media was removed from the plates and they were covered with a thin layer of sterile PBS. Approximately 5µl of trypsin – EDTA was drawn up into a 1-200µl pipette tip. Under the magnification of an inverted light microscope, a colony was localised, a small volume of trypsin expelled and then the colony gently aspirated into the pipette tip. The content of the pipette tip was combined with a further 100µl of trypsin and the colony gently dispersed into a single cell suspension by repeated gentle pipetting prior to transfer to a 24 well plate ($1cm^2$ surface area). Cells from the colonies were gradually expanded into larger tissue culture flasks so that they could be harvested for western blot analysis and frozen for further studies. Colonies were kept under antibiotic selection until they were expanded into $25cm^2$ flasks (a minimum of seven days post colony isolation) and were thereafter maintained in the usual culture media for each cell line.

### 2.8.9 Detection of EGFP Fluorescence

Cells were harvested in the usual manner and then seeded into an eight chamber microscope slide (Lab-Tek™ - Chamber Slide™, NUNC, Fisher) at a confluence of approximately 25%. Twenty four hours later the chambers were removed and a microscope cover slip placed on the slide. Cells were visualised using a fluorescent inverted microscope with a FITC filter and images recorded using an attached digital camera.

## 2.9 Cell proliferation experiments

Cells were plated onto 6 well plates (area 9.5cm$^2$ / well) in their usual growth media aiming for a confluence 24 hours later (Day 0) of approximately 10%. Cells were harvested every 24 hours and total cell count per well ascertained using a haemocytometer chamber and / or an automated particle analyser, aka a coulter counter (Beckman Coulter – Z series, Beckman Coulter Inc.).

Using the haemocytometer, the freshly harvested media (2ml per well) was pipette agitated and then applied to the haemocytometer slide underneath a tightly adherent cover slip, monitored by checking for the presence of Newton's rings. Cell numbers in four separate 1mm x 1mm squares were manually counted including any cells overlapping the top and left borders and excluding cells crossing the bottom and right borders. The mean of these four totals was then multiplied by 20,000 (haemocytometer chamber volume = 0.1mm$^2$), providing a value for the total number of cells harvested from each well. Four wells were analysed for each condition and time period and the mean value of these plotted as a proliferation curve with the standard error of the mean forming the error bars.

The coulter counter was maintained, flushed and calibrated as per manufacturer's instructions. Cells were harvested in the smallest volume needed for adequate trypsinisation. 0.5ml of these harvested cells formed the metered volume for the Coulter analysis. This cell suspension was combined with 10 ml of isoton II diluent (Beckman Coulter inc.) to fill the accuvette (Beckman Coulter inc.) and using a 100μm aperture tube all particles between 8 and 20 μm were filtered and counted. This produced a value expressed as [cells / ml] which was then multiplied by the appropriate total initial harvested volume (0.5ml – 5ml), as necessary. The average of three wells per condition was plotted with error bars as above.

For the initial proliferation experiment (see section 6.2) cell counts were performed using both a haemocytometer and an automated flow cytometer to ascertain the accuracy of the automated system for this given cell population. By comparing the total cell number for the same series of experimental conditions it would be possible to determine if the faster coulter counter method could be utilised accurately in the larger scale cell proliferation studies to follow (section 6.3).

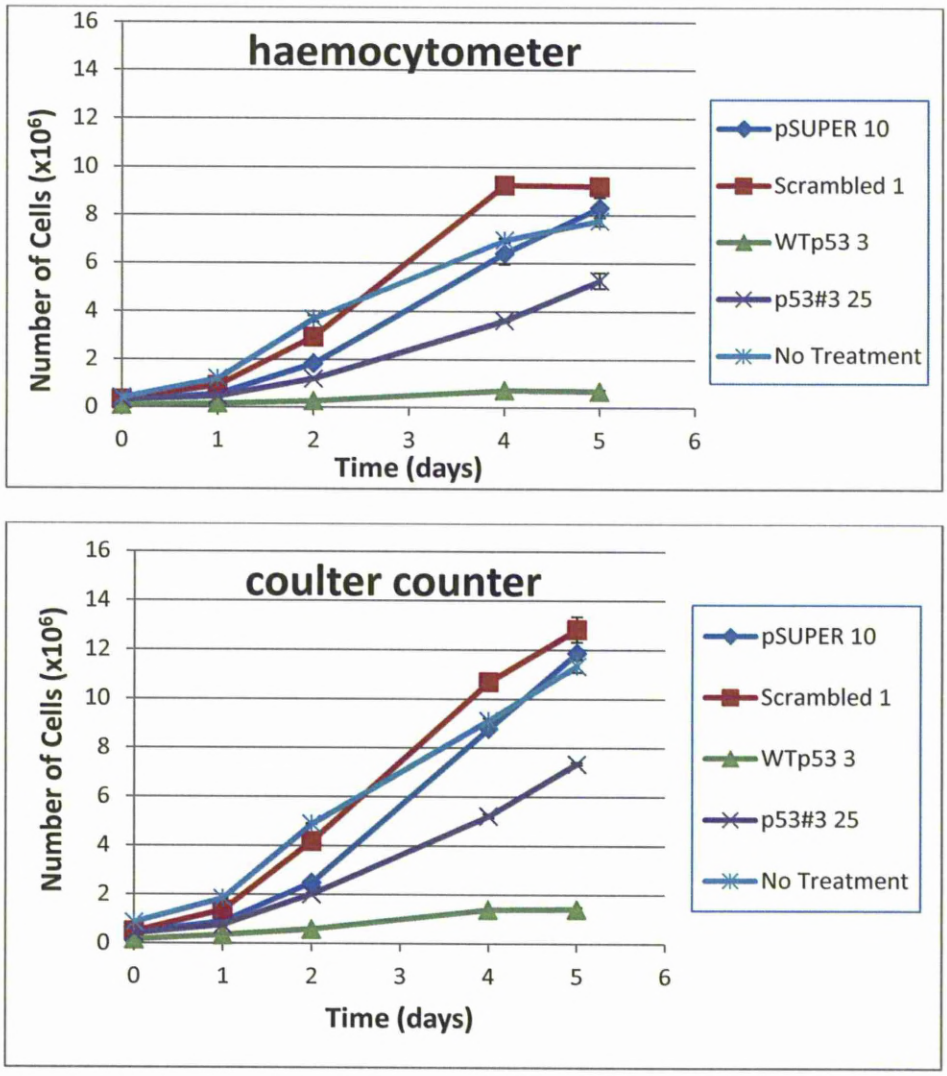



***Figure 2.8: Comparison of cell number counts with the haemocytometer verses the coulter counter method.***

The graphs show that similar results are achieved with the two cell counting methods confirming that the coulter counter is an acceptable method counting cell numbers in the further proliferation experiments.

## 2.10 Chemotherapeutic agent toxicity assays

5-fluorouracil (5-FU) (Sigma, $C_4H_3FN_2O_2$, MW = 130.8g) was dissolved in DMSO (0.065g /ml) [0.5M] stock and stored at 4°C. This was further diluted to [10mM] in DMSO and added to the appropriate tissue culture media volume (2ml / well) to obtain final working concentrations of 1 to 20μM 5-fluorouracil.

In order to try and avoid any adverse effects from DMSO toxicity, the volume of DMSO / 5-FU added to the media was kept at a minimum (0.02%), total DMSO volume added was equal for all [5-FU] and a DMSO only control was also examined.

Oxaliplatin (LC Laboratories, $C_8H_{14}N_2O_4Pt$, MW = 397.29g) was dissolved in $H_2O$ (0.4g/ml) [1M] stock and stored at 4°C. This was diluted in water to [10mM] and added to the media to obtain [Oxaliplatin] 0.25μm – 10μm.

Cells were seeded in 6 well plates as previously, aiming for starting confluencies of 10-20% 24 hours later. Tissue culture media was removed and media containing the chemotherapeutic agents applied and left on the cells for the duration of the experiment. Cells were harvested and counted at 0, 24, 48 and 72 hours using the Coulter analyser and cell proliferation graphed and analysed using the methods discussed in section 2.9.

# Chapter 3. Establishing baseline levels of p53 and 'p53 pathway' proteins

## 3.1 Determining the basal levels of p53 protein expression in our panel of colorectal cell lines

Before embarking on studies of the effectiveness of RNA interference, it was necessary to perform some simple and basic analyses of the colorectal cells lines to be studied, since this was the first time that colorectal cells had been examined in our laboratory. This would fulfil the dual purpose of confirming that the phenotypic p53 status matched the published data (see section 2.2) and allow refinement of tissue culture techniques with specific reference to these new cell lines, as detailed in section 2.3. Therefore initial experiments involved western blot analysis of basal p53 protein expression levels in lysates prepared from a panel of colorectal cancer cell lines to determine the steady state levels of p53 expression.

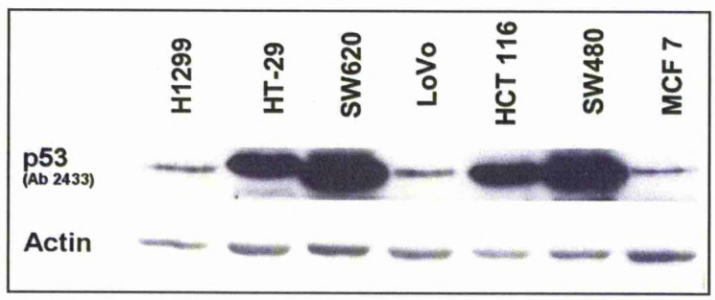



***Figure 3.1: Determination of endogenous p53 expression levels in a panel of colorectal cells***

*Figure 3.1and 3.2: Cell lysates were prepared with protein concentrations of 50µg/20µl and were analysed alongside15µl of broad range pre-stained molecular weight marker (New England Biolabs) by western blot using the Ab 2433 p53 primary antibody (Abcam), as outlined in section 2.4. Actin was used as a reference to confirm even sample loading. The breast cell line MCF 7 was also analysed alongside the colorectal samples since they have been used frequently in our laboratory and were known to express low levels of wild type p53 and they therefore*

*provided a positive control for p53 detection. The non small cell lung cancer H1299 cell line was used as a negative control as they are known to be p53 null.*

Figure 3.1 demonstrates very high levels of p53 protein expression in the mutant cell lines SW480 and SW620 consistent with their p53 point mutation(s) and over-expression. HT-29 cells displayed relatively lower expression, despite their mutant status, but interestingly the band for p53 appeared at a slightly higher apparent molecular weight than in other cell lines. HCT-116 cells exhibited relatively high levels of p53 expression considering their wild-type status. In comparison, the wild-type LoVo cell line expresses relatively low levels of p53, comparable with MCF7 cells. Of concern, the H1299 cells displayed a band at approximately 53kDa, despite their p53 null genotype. Therefore subsequent western blots were repeated using the p53 primary antibody DO-1 (Ab 6), rather than AB-2433, which successfully eliminated this non-specific band as demonstrated in Figure 3.2.

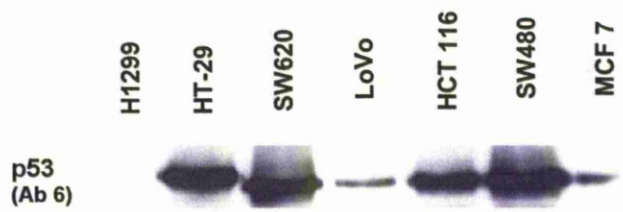



***Figure 3.2: Determination of endogenous p53 expression levels in a panel of colorectal cells using primary antibody DO-1 (Ab 6)***

## 3.2 Basal protein levels in an array of 'p53-pathway' proteins

In the next set of experiments basal levels of various proteins involved in p53 pathway were examined; genes located upstream (p14 $^{ARF}$), those involved in regulation of p53 stability/activity respectively (Mdm2 / MDMX) and genes encoded by downstream transcriptional target genes (CDKN1a / PirH2) were subjected to western blot analysis in our panel of colorectal cells. The results of these western blots are summarised in figure3.2:

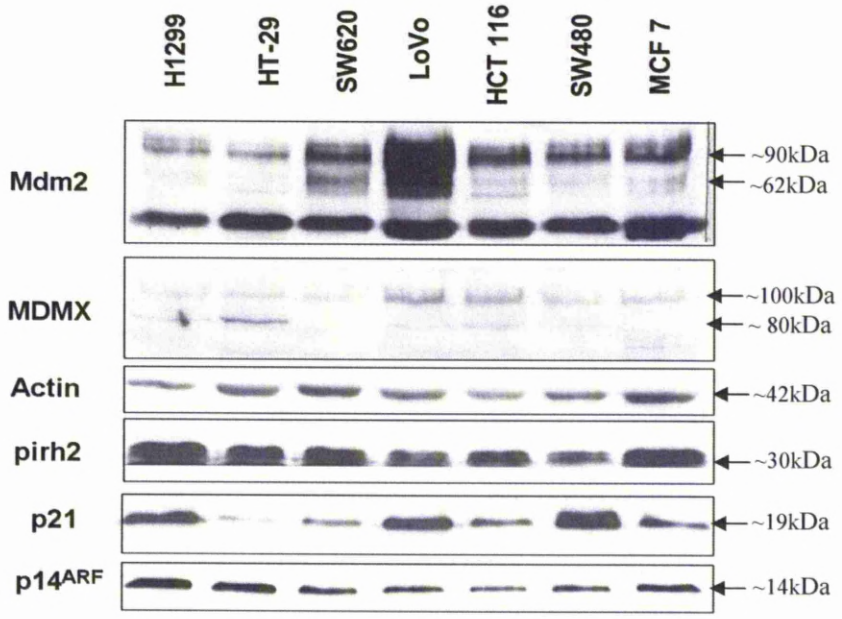



***Figure 3.3: Basal protein expression of a sample of 'p53-pathway' proteins***

*Cell lysates were prepared as outlined in section 2.4.1 and 50µg/20µl of protein product was analysed alongside 15µl of broad range pre-stained molecular weight marker (New England Biolabs), by western blot. The DO-1 p53 primary antibody (Oncogene/Merck) was utilised alongside the other primary antibodies detailed in Table 2.3. The approximate relative molecular weight of the proteins and any visualised break down products, as expected from the published literature is presented to the right of the displayed plot. MCF 7 and H1299 cell lines were added as control samples as per Figure 3.1.*

In Figure 3.3 it was notable that the wild-type LoVo cell line appeared to exhibit relatively high levels of MDM2 expression compared with the other cells analysed. MDMX protein expression appeared relatively high in both wild-type cell lines, as well as the mutant HT-29 cells, when compared with MCF 7 cells and our laboratory's previous experience in other cell lines. There was no apparent correlation between p53 wild-type or mutant status and the expressed levels of pirh2, p21 or $p14^{ARF}$.

# *Chapter 4. Evaluation of p53 siRNA specificity and efficacy*

## 4.1 The effects of siRNA mediated p53 and MDM2 down regulation on protein expression levels and p53 mediated transcriptional activity.

Mutant p53 cell lines typically have very high levels of expression of the mutant protein and this finding has already been documented in our basal level experiments (section 3.1) within our chosen colon cancer cell lines. Therefore prior to targeting mutant p53 cell lines using RNA interference, which may prove troublesome due to these high protein expression levels, an initial experiment was designed that would target wild-type p53.

SiRNA oligonucletides, as detailed in section 2.6.1, were utilised to target wild-type p53 and MDM2 sequences in transient suppression experiments on three isogenic variants of HCT 116 cells (+/+, +/- and -/-). The p53 siRNA sequence chosen was selected because a previous study had suggested it had shown specificity for wild-type verses mutant p53 protein [244].These isogenic variants allowed the specificity and magnitude of p53 suppression to be studied, since the heterozygous and null phenotypes could be compared against the observed effect in the parent cell line. The results of siRNA mediated wild-type p53 suppression in HCT 116 cells and their heterozygous and null phenotype derivatives, are shown in Figures 4.1 and 4.2:

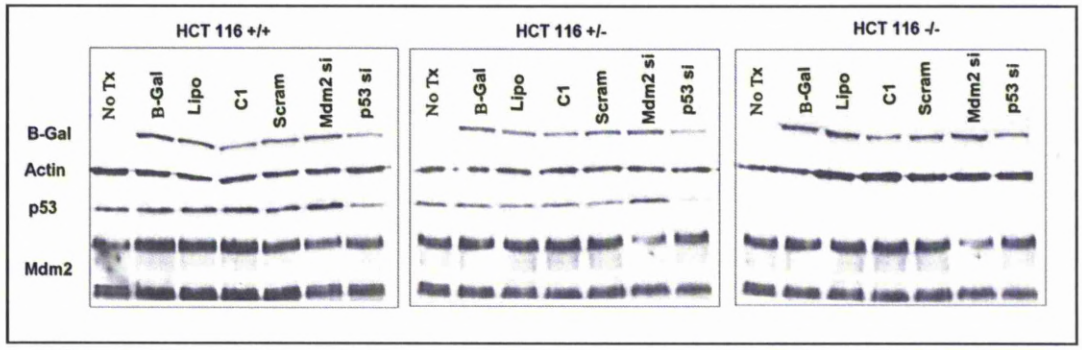



***Figure 4.1: siRNA mediated suppression of p53 protein levels in HCT 116 cells and derivatives***

*HCT 116 cells and their heterozygous and null derivatives underwent transfection with the p53 and MDM2 siRNA sequences detailed in 2.6.1. A scrambled siRNA sequence (produced from a rearranged MTBP siRNA) and a commercially purchased control sequence (Daramacon) C1 were used as negative controls. A lipofectamine only treatment group was also studied to account for transfection reagent mediated toxicity.*

*The cells were plated in 6 well tissue culture plates (each well with an area of 9.5cm$^{2)}$ and 24 hours post plating (confluency ~40%), the cells were underwent transfection with the luciferase reporter plasmid (pp53-TA-Luc) and a β-galactoside expressing plasmid.1.18μg of pp53-TA-Luc plasmid and 0.42μg of B-Gal plasmid per well was applied with the transfection reagent GeneJuice (Novagen), used at a volume of 4.2μl per well. These reagents were combined with 100μl of serum free media and applied to the cells for 4 hours at which point this media was removed.*

*The siRNA, at [40nM], was combined with 400μl Opti-mem media (invitrogen) and 4μl Lipofectamine 2000 (Invitrogen) per well. 1.6mls of serum free media was added to each well and incubated for a further six hours. This media was replaced at 6 hours to negate the possible toxic effects of prolonged exposure to the Lipofectamine reagent. 48 hours after the application of the siRNA solution the cells were harvested with three wells per condition collated to allow 50μg/20μl of protein to be loaded for western blot analysis as per section 2.4.*

Figure 4.1 demonstrates that a detectable reduction in p53 protein level expression was obtained with the p53 targeted siRNA, in both the parent and heterozygous cell lines and thus the siRNA sequence appears to work in principle. MDM2 suppression was also visualised on the western blot, though the proportional reduction with this siRNA sequence appeared less then that seen with the p53 siRNA sequence. The p53 protein levels increased in response to the suppression of MDM2 as one would expect in wild-type cells with negative inhibition from the p53 / mdm2 feedback mechanism.

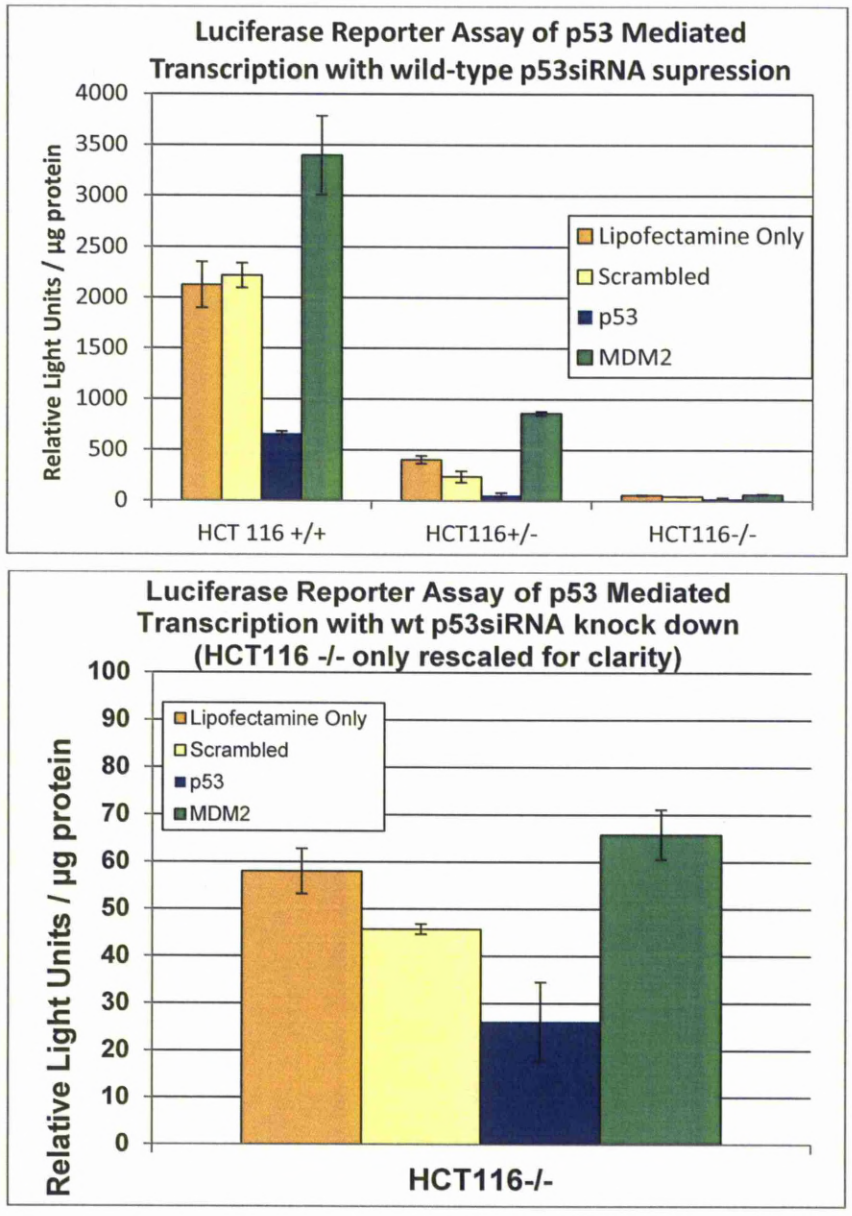



***Figure 4.2: The effects of siRNA mediated suppression on p53 transcriptional activity in HCT 116 cells and derivatives***

*The siRNA transfection was undertaken as detailed for Figure 4.1. 3 of the wells per condition were used as separate replicates for the luciferase reporter assay. A Glo-lysis luciferase reporter assay kit (Promega) was used. The cells were then lysed with 500µl of the Glo-lysis buffer and centrifuged at 16,100 x rcf for 3 mins to pellet cellular debris. The supernatant was decanted and 50µl of this was added to 50µl of the luciferase reagent. This was immediately placed in a Turner Design 20/20 Luminometer and readings were taken for a thirty second period following an eight second pre-reading pause. Results were recorded in relative light units (RLU). The samples were normalised to RLU / µg protein using a Bradford assay to estimate protein concentrations (2.4.2). The mean of the three normalised results was plotted with the standard error of the mean (SEM) used and presented as the Y-axis error bars (see section 2.6.1). The same data from the HCT116 -/- derivative is repeated in the second histogram after re-scaling to improve clarity.*

Figure 4.2 shows the p53 wild-type (+/+) cells demonstrate a high level of luciferase activity, concomitant with functional p53 transcriptional activity. On siRNA suppression of the wild-type protein, p53 transcription was reduced to 30% of original activity in the wild type cells. This confirms the successful suppression witnessed with western blot analysis as discussed in figure 4.1. The p53 transcriptional activity is enhanced by transfection of the MDM2 siRNA as we would expect in a functional p53/MDM2 feedback mechanism.

The HCT 116 +/- derivative demonstrates p53 transcriptional activity at less than 25% of the homozygous parent cell line however the pattern of response to p53 and MDM2 siRNA mediated down regulation is as seen and described in the +/+ cells above.

The basal levels of transcriptional activity in the HCT 116 -/- derivative were proportionally very low as expected. However, on re-scaling the data in the second histogram, these low protein levels still appear to be transcriptionally active, since they are down regulated with the p53 siRNA and upregulated with the MDM2 siRNa, as seen in the other derivatives. The p53 homozygous -/- cells are not truly p53 null, rather they contain a p53 ΔN40 mutation on both alleles. This could account for the apparent residual activity seen in figure 4.2.

The next series of experiments was designed to study the effects of siRNA mediated p53 down regulation on our panel of colon cancer cell lines expressing mutant p53. The same wild-type p53 siRNA sequence was used as outlined above (see also section 2.6.1).This targets a sequence outside the mutated regions of our mutant p53 expressing cell lines [244] as cited below and hence should be able to suppress transcription of the mutant protein expression in these cell lines.

P53 mutations in our colon cell lines:

HT29 R273H mutation

SW480 R273H mutation

SW620 R273H and P309S mutations.

The first aim of this experiment was to see whether any detectable reduction in the very high levels of mutant p53 levels expressed by our cell lines, as demonstrated in Figure 3.1, could be achieved using siRNA mediated suppression.

The second aim of the experiment was to see if the mutant p53 cell lines retained any residual p53 transcriptional activity and if so would this be regulated by p53 / MDM2 down regulation, as witnessed previously in the HCT116 +/+ wild-type cell line (Figure 4.2).

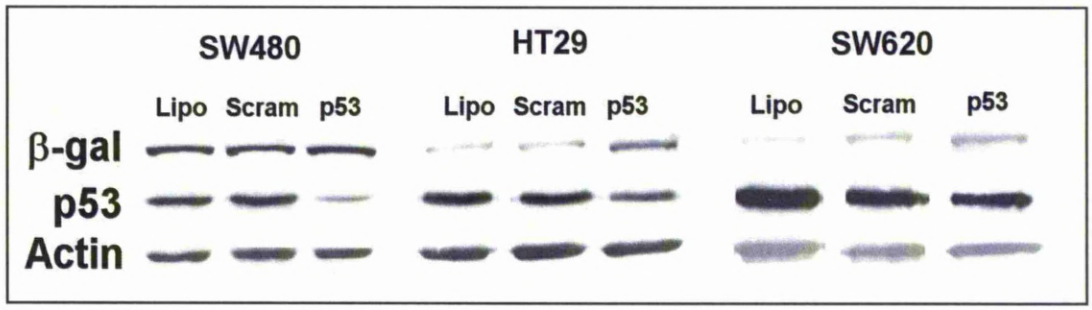



***Figure 4.3: siRNA mediated knock-down of p53 in mutant colorectal cell lines.***

*The siRNA mediated suppression was conducted with the same siRNA sequences and method previously discussed in figure 4.1. Again 50μg/20μl of protein was analysed for western blot analysis with results presented for the p53 and scrambled siRNA transfections alongside the lipofectamine only control.*

In figure 4.3 two of the mutant cell lines show a demonstrable reduction in mutant p53 levels with siRNA transfection, though this cannot be confirmed in the SW620 cell line due to the overexposed blot. The high levels of expression of the mutant p53 protein in all of 3 cell lines resulted in the necessity to use very low concentrations of p53 primary antibody to obtain appropriate images (0.3μg/ml compared with 3μg/ml for wild type protein). Even with dilution the required exposure time for the western blots was very short. This demonstrates the magnitude of mutant p53 expression when compared with the wild-type p53 levels.

The proportion of suppression of p53 protein levels by the siRNA sequence appears less than that witnessed in the HCT 116 wild-type cells. This could represent saturation of the siRNA by the over-expressed mutant protein, or it could be due to the mutant p53 protein having a longer half life than the wild-type p53 and therefore being more resistant to this transient siRNA suppression. Also the mutant cell lines appeared quite difficult to transfect compared with the wild-type HCT116 +/+, as suggested by the weak β-Gal signal even on prolonged film exposures, especially in the SW620 and HT-29 cell lines.

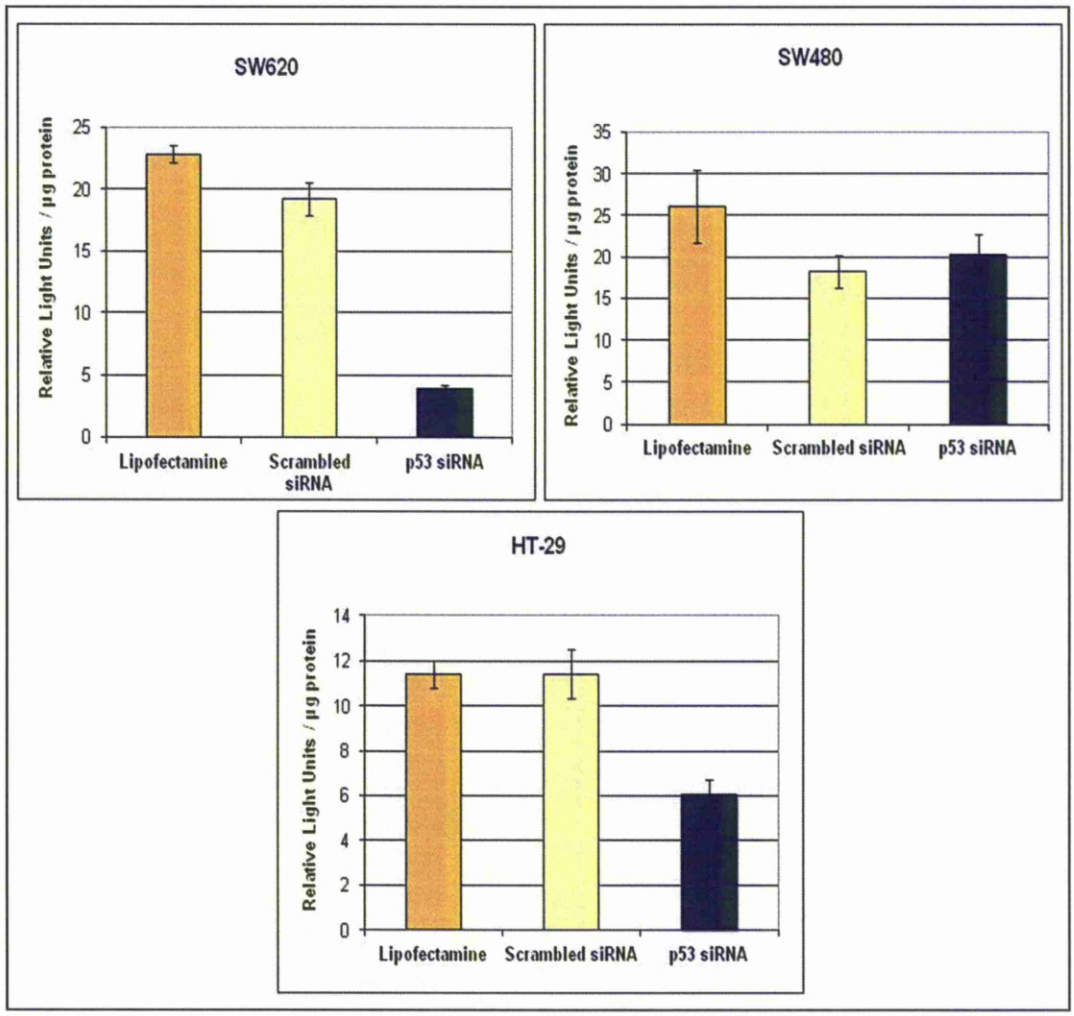



***Figure 4.4: p53 transcriptional activity with p53 siRNA knock-down in SW620, SW480 and HT-29 cell lines***

*The Luciferase Reporter Assay was conducted as previously detailed in Figure 4.2 except that lysis of the mutant cell lines needed only 300µl of Glo-lysis buffer per condition.*

Figure 4.4 demonstrates that the level of luciferase activity and hence p53 activity is far lower (<3 %) in all of the mutant cell lines than in the wild-type, the Relative Light Units on the y axis being less than 30 in Figure 4.4 compared with greater than 2000 in Figure 4.2. However, the results indicate that SW620 and HT-29 may retain a low level of p53 transcriptional activity despite their mutant p53 status. Any residual p53 activity in the SW480 cells is not obviously affected by p53 siRNA knockdown.

## 4.2 The effects of mutant specific siRNA nucleotides on p53 protein expression and transcriptional activity.

The next experiment was designed to address the question of whether any selectivity of mutant verses wild-type p53 suppression could be achieved by designing siRNA sequences against the specific R273H mutation harboured by all 3 of our mutant p53 colon lines, in addition to the PANC1 cells, see section 2.2.1. Hence three 19 base pair siRNA sequences were designed to target the specific R273H p53 DNA binding domain missense point mutations found in the above mentioned selected cell lines. These were transfected into both wild type and mutant cell lines, as previously, and were compared with the wild type targeting siRNA for efficacy and specificity.

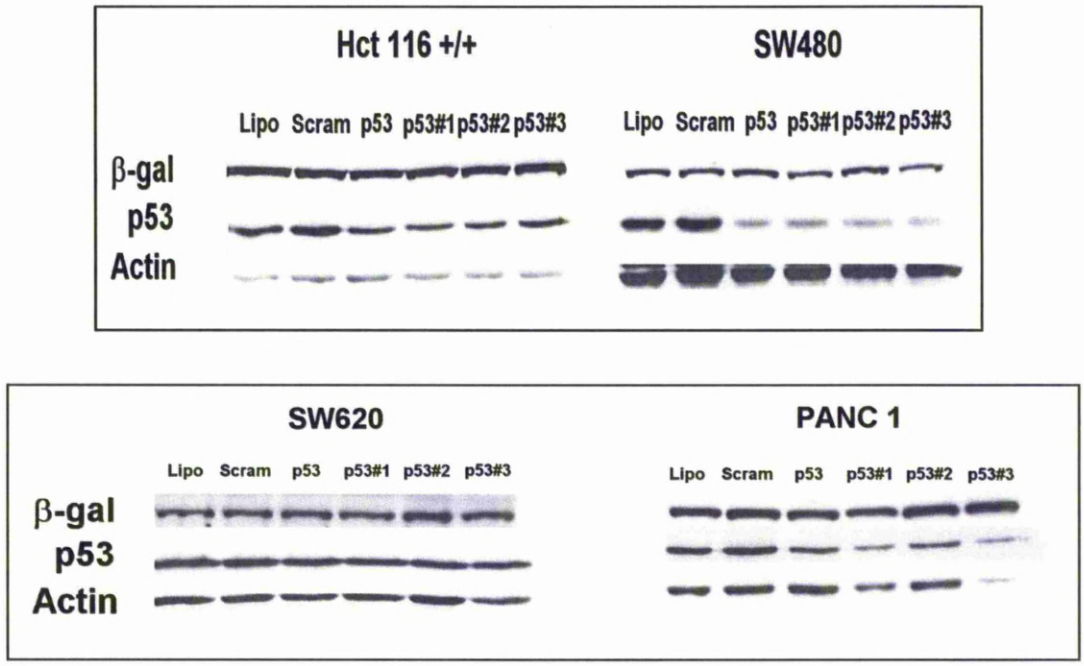



***Figure 4.5: The effect of mutant p53 siRNAs on wild type and mutant p53 protein expression***

*siRNA transfection was undertaken as outlined in Figure 4.1 and western blot analysis performed as per section 2.4.*

Figure 4.5 demonstrates successful down regulation of p53 in the HCT116 +/+, SW480 and PANC1 cell lines, though the latter needs careful interpretation due the uneven actin levels and hence protein loading demonstrated. There is no visable suppression of p53 in the SW620 cell line, which repeats the finding suggested in Figure 4.3. It may be that this cell line may be inherently more difficult to transfect.

There is a suggestion of some specificity with the mutant p53 siRNA number 3 (p53#3). It appears to reduce the p53 levels less than the wild-type p53 in the HCT116 wild-type cell line whilst reducing the p53 levels more in the SW480

mutant cell line than the wild-type targeted sequence. There was no such specificity demonstrated for the other two p53 specific siRNAs (p53#1 and p53#2).

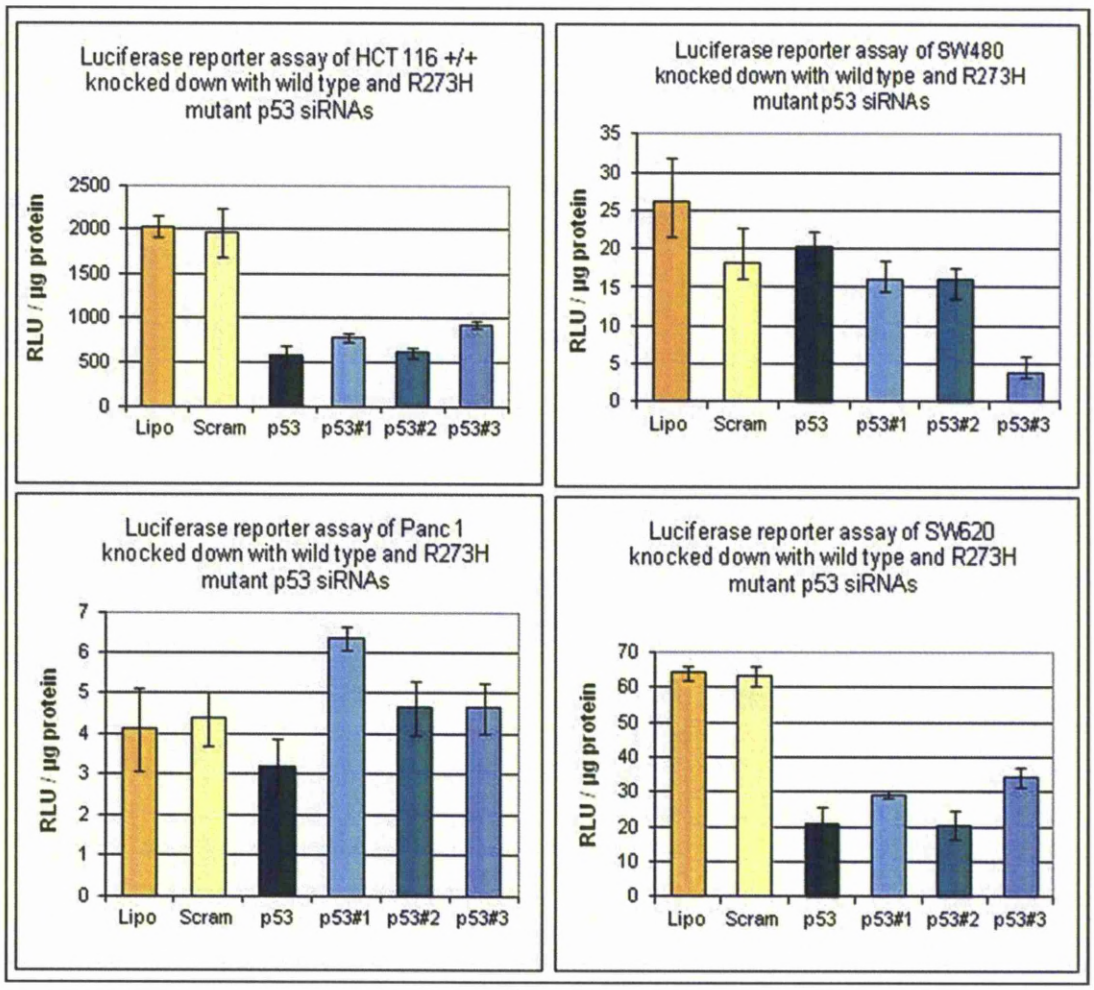



***Figure 4.6: The effect of mutant p53 siRNAs on wild type and mutant p53 mediated transcriptional activity***

Figure 4.6 confirms that the wild-type p53 is less suppressed in the HCT116 +/+ cell line with the p53 mutant specific siRNA #3, than with the wild-type p53 siRNA. Though this does not apply to the other two R273H specific siRNAs. In 2002, Martinez et al [118] showed discrimination between wild type and mutant p53 with a single base pair alteration in siRNA was possible. However, other groups have since shown that as little as 11 or even 7 bases of contiguous homology in a siRNA sequence may be sufficient for inhibition [119][120]. In the example of endogenous miRNAs only 6 or 7 bases with perfect homology are required for the seed sequence to enable targeting of the miRNA.

In summary, it may be possible to design an siRNA that is somewhat selective for a specific p53 mutant but this may not be as trivial or as reliable as some previous studies (Martinez) have suggested [244].

## 4.3 In-situ β-galactosidase assay to assess transfection efficiency

The aim was to ascertain whether the relatively poor degree of p53 knock down observed in the p53 mutant colorectal cell lines thus far was the result of cells which are inherently resistant to transfection, rather than a deficiency in the experimental method. The use of a β-galactosidase assay can only provide a general guide as to the ease of transfection of the cell line, since siRNA transfection rates in each cell line may not necessarily equate with that of β-galactoside plasmid uptake. However if β-galactosidase uptake was globally poor this may raise concerns regarding the experimental method.

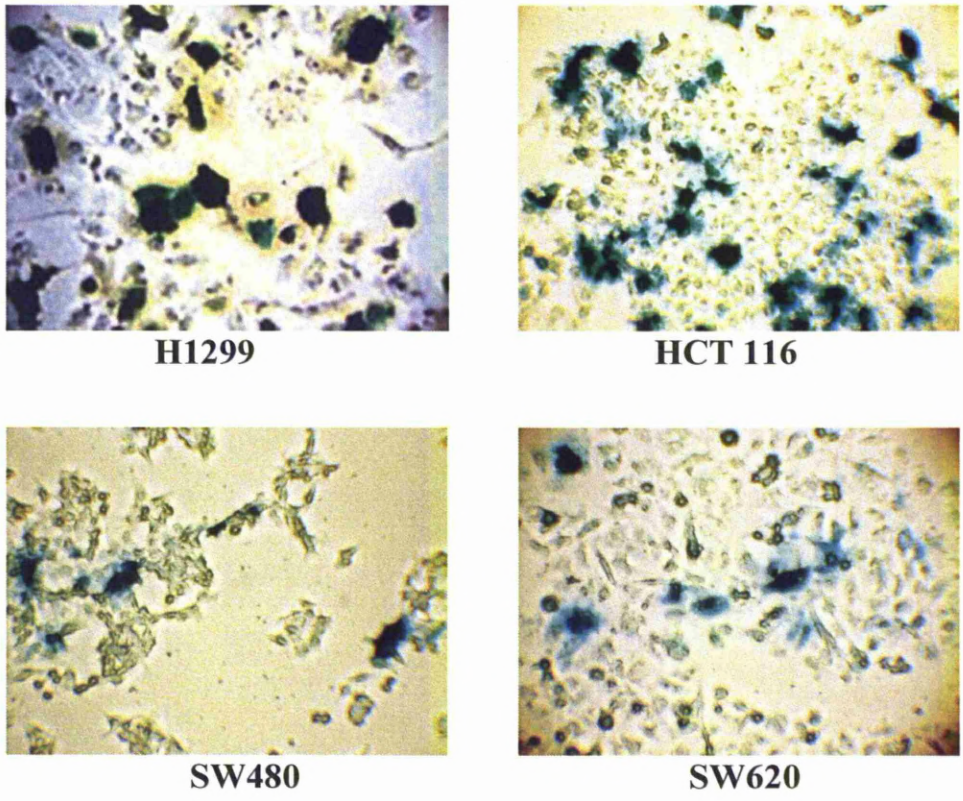



***Figure 4.7: In situ β-Galactosidase assays***

*Cells were plated the day before transfection into one 10cm dish per cell line, aiming for ca. 40% confluence on the day of transfection. β-galactoside and pp53-TA-Luc vectors were transfected as outlined previously in section 2.6.2 . Twenty-four hours later the media was removed, the cells washed with sterile PBS twice and 10ml of fixing buffer (2.1.20) added per dish and left at room temperature for 15 minutes. This was removed and the cells again washed with PBS, prior to the application of 5-10ml per dish of substrate buffer (2.1.21). The cells were incubated at 37°C for 3 – 8 hours, after which the substrate buffer was removed and the plates washed twice with water. Once dried, the plates were visualised by microscopy (magnification 100x) to assess the number of positively transfected (blue) cells and a photographic record made.*

Figure 4.7 compares β-galactoside transfection in 3 colorectal cell lines with that of H1299 cells, in which transfection efficiencies of 30-40% had been achieved routinely by other members of our laboratory [121]. It demonstrates approximately 30% of cells staining positively for β-galactoside in the H1299 and p53 wild-type HCT 116 cell lines. This suggests experimental efficiency to be comparable with previous expectations. However under the same experimental conditions, B-Gal

uptake was only 5-10% in two of the mutant p53 colon cell lines (SW480 and SW620). This confirms that these cell lines may be more resistant to transfection with the β-galactoside plasmid and possibly also to siRNA transfection.

## Optimisation of siRNA mediated transfection.

Due to the difficulty in obtaining efficient p53 knock down in the mutant colorectal cell lines, a range of experimental conditions were altered to try and optimise siRNA transfection efficiencies. The wild type HCT 116 +/+ were utilised as a positive control, since a 50% knock down in this cell had proven achievable, alongside the SW620 mutant, which had proven the most difficult cell line in which to knock down the mutant p53 protein. The variables considered were concentration of siRNA, ratio of transfection reagent to siRNA, the time course of knock-down effectiveness of siRNA and these are considered below.

### 1) [siRNA]

The Lipofectamine 2000 protocol (Invitrogen) suggests using 1 μl of Lipofectamine 2000 per 20pmol siRNA as a guide ratio. It recommends further optimisation by considering siRNA: Lipofectamine 2000 ratios of 10 – 50 pmol siRNA: 0.5 – 1.5μl of Lipofectamine 2000.

The standard technique of [40nmol] of siRNA (4μl of Lipofectamine 2000 with 80pmol siRNA in 2 mls of media = 1μl Lipofectamine 2000 per 20pmol siRNA) was compared against siRNA [100nmol] (50pmol siRNA / μl of Lipofectamine 2000).

**2) Volume of Lipofectamine 2000**

1µl and 2µl of Lipofectamine 2000 per 20 pmol siRNA using [40nmol] siRNA

**3) Duration of exposure**

Cells were harvested at 24, 48 and 72 hours post transfection.

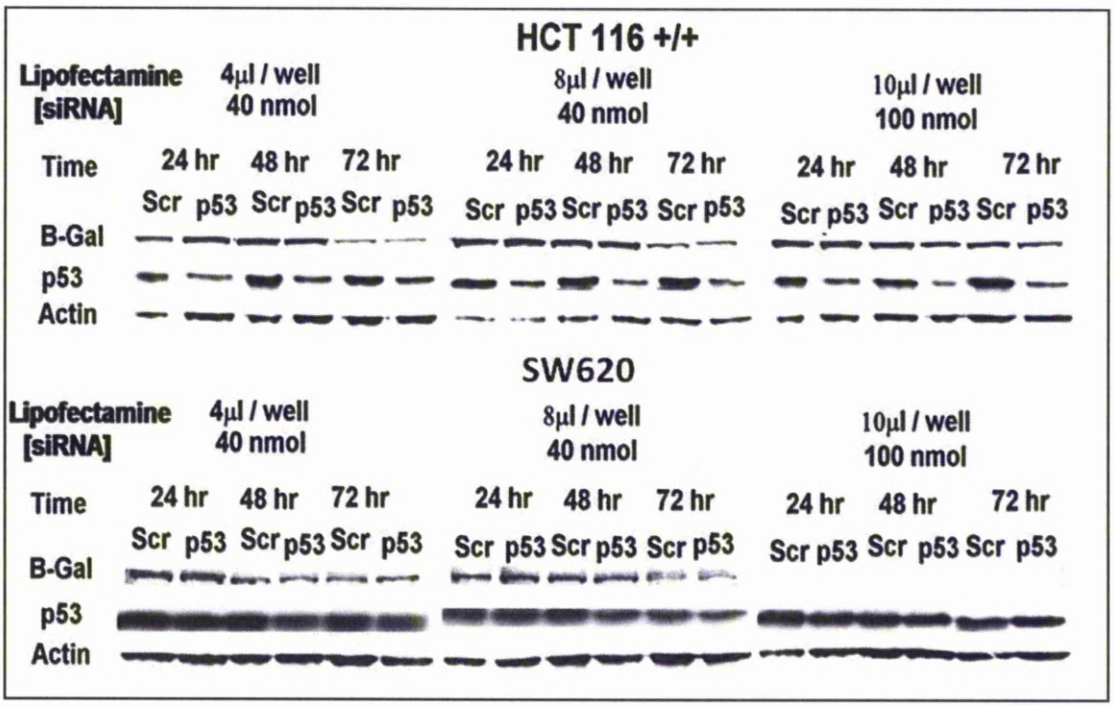



***Figure 4.8: Western blots of siRNA delivery optimisation experiments***

*The effects of the wild-type p53 siRNA were compared against the scrambled control by analysis of protein concentrations on western blot for the conditions outlined in 4.2 above.*

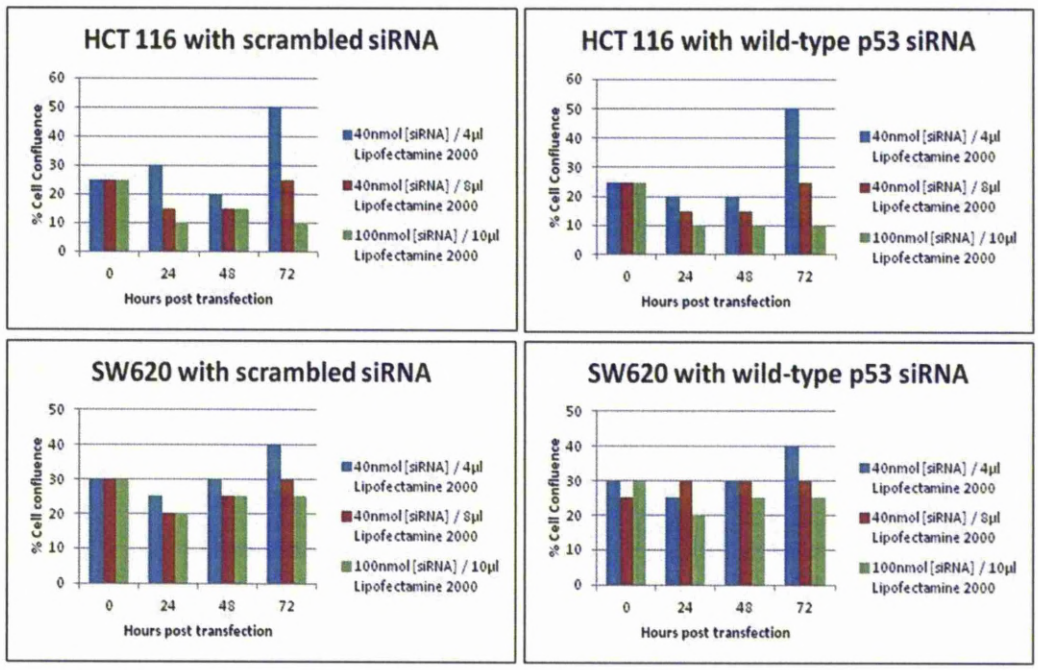



***Figure 4.9: Histograms demonstrating cell confluence with siRNA optimisation experiments***

*The % cell confluence in the tissue culture flasks was studied by microscopy every 24 hours for the varying optimisation conditions outlined in section 4.2. This was to assess for any adverse toxicity reducing cell viability over the duration of the experiment, since lipofectamine 2000 is established as having a cytotoxic effects.*

Figure 4.8 demonstrates only a marginal improvement in suppression with any of the variables studied. It appeared that harvesting at 48 hours post transfection gave maximal suppression which was consistent with previous practise. The higher siRNA concentrations and Lipofectamine volumes improved knock-down slightly in the HCT116 cells, but this was associated with reduced cell confluence, as shown in Figure 4.9, suggesting a higher level of toxicity and resulting in lower total protein yields. None of the above measures resulted in a significant p53 down regulation in the problematic SW620 mutant cell line (Figure 4.8), although SW620 did appear more resistant to the potential toxicity of higher siRNA concentrations and Lipofectamine 2000 volumes than the other cell lines studied.

# *Chapter 5. Stable knock down with the pSUPER neomycin / GFP vector*

## 5.1 Introduction

The limitations of siRNA mediated knock down are its transient nature and the fact that efficacy of target gene suppression is dependent on the efficiency of transfection achievable in any given cell line. Therefore, in order to study the effects of p53 mediated suppression on cell growth and response to chemotherapy in the mutant p53 colon cancer cell lines, it was decided that stable shRNA expressing clones would need to be developed. These can provide for the stable suppression of the target gene in question (Brummelkamp)[248], rather than the limitation of a few days experienced with siRNA technology. The availability of a commercial vector, with a neomycin resistance gene, should also help overcome poor transfection efficiencies, since only clones with plasmid DNA integrated into their genome will survive the antibiotic selection process.

## 5.2 Transient transfections

Prior to plating the pSUPER transfected cells for growth under antibiotic selection, samples of the cells were harvested for western blotting to assess whether our shRNA expression mechanism appeared to be functional. Levels of p53 mediated knock down, as well as detection of EGFP, were used to confirm that some functional plasmid had undergone transcection.

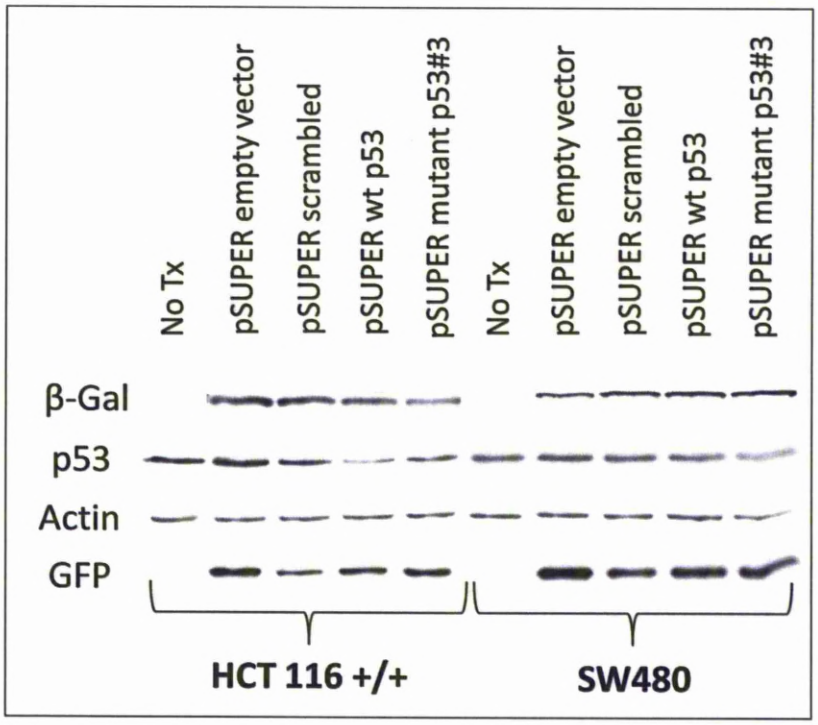



***Figure 5.1: Results of transient pSUPER transfection on p53 protein expression***

*The pSUPER empty vector and those containing the scrambled, wild-type p53 and p53#3 mutant inserts were transfected into HCT116 +/+ and SW480 cells as outlined in sections 2.8.1 – 2.8.6 and 2.8.8. The cells were harvested and lysed 24 hours later and 50µg/20µl of the protein product was run on an SDS page for western blot analysis with the DO-1 (Ab 6) p53 primary antibody (Section 2.4).*

Figure 5.1 demonstrates a degree of p53 knock down in the HCT 116 cell line even in these transient studies, along with detectable levels of GFP expression, both of which were reassuring that the plasmid was functioning effectively. It was also encouraging that the mutant targeted vector appeared to be showing some specificity, with less suppression of the wild type p53 protein than with the wild type shRNA expressing vector. The SW480 mutant cell line showed only a marginal reduction in p53 protein levels, but again there appeared to be potential specificity with the wild-type shRNA expressing pSUPER showing no demonstrable effect on the mutant p53 protein levels.

## 5.3 EGFP expression in pSUPER PANC 1 clones

Initially the pSUPER plasmid and derivative constructs (expressing wild-type and mutant p53 shRNA or scrambled control), underwent transfection into PANC 1 cells, since they could undergo successful transfection more readily than the p53 mutant colorectal cell lines.

Once specific clones had been obtained from selected cultures by picking / isolating individual colonies grown under neomycin selection, it was possible to confirm true transfection of the plasmid, as opposed to acquisition of antibiotic resistance, by viewing the cells with a fluorescent microscope. Clones that had undergone positive transfection should be expressing the enhanced green fluorescence protein and therefore should glow luminescent green in response to an ultra-violet stimulus. Examples of PANC 1 cells exhibiting such a response are shown in Figure 5.2:

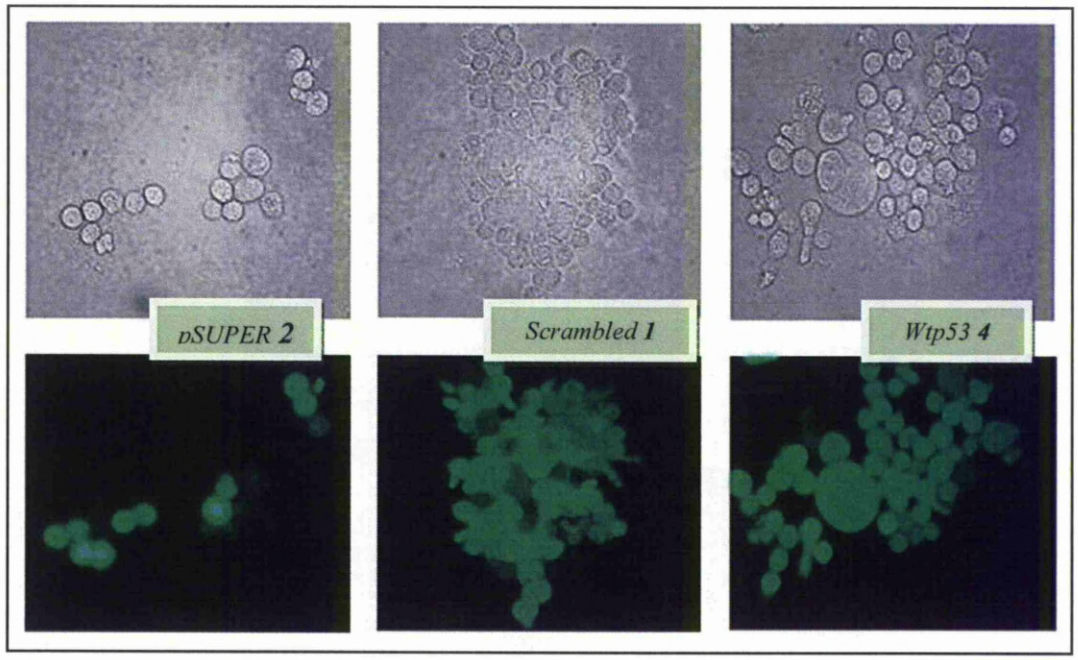



***Figure 5.2: Examples of EGFP expression from PANC 1 pSUPER clones***

*Cells harvested after antibiotic selection (Sections 2.8.8 - 2.8.9) were seeded into an eight chamber microscope slide (Lab-Tek™ - Chamber Slide™, NUNC, Fisher) at a confluence of approximately 25%. Twenty four hours later the chambers were removed and a microscope cover slip placed on the slide. Cells were visualised using a fluorescent inverted microscope with a FITC filter and images recorded using an attached digital camera. The images are shown at original magnification (x100) and represent three of the clones that expressed high levels of GFP protein namely pSUPER 2, Scrambled 1 and wild-type p53 4. The plain microscopy image for each clone is displayed superiorly with the associated UV fluorescence image beneath. Levels of EGFP expression were variable between clones and within cells of one clonal population as discussed further in section 5.5.*

## 5.4 Confirmation of mutant p53 knock-down in Panc 1 Clones.

Even when utilising neomycin selection and then GFP expression to detect positive transfectants, the levels of siRNA expression and gene knock-down could still vary widely among the individual clonal populations pSUPER transfected cells that survive antibiotic selection may not have a significant reduction in expression of the target gene. Instead, they may have found a way to mitigate the effects of a reduction in the target gene expression by compensating in another fashion, e.g. by shutting

down expression of the shRNA or simply though rearrangement of the plasmid such that the neomycin cassette is retained but the shRNA cassette is disrupted. Therefore multiple clones were isolated for each vector; these were then screened using western blot analysis, to determine whether the desired reduction in target gene expression was observed.

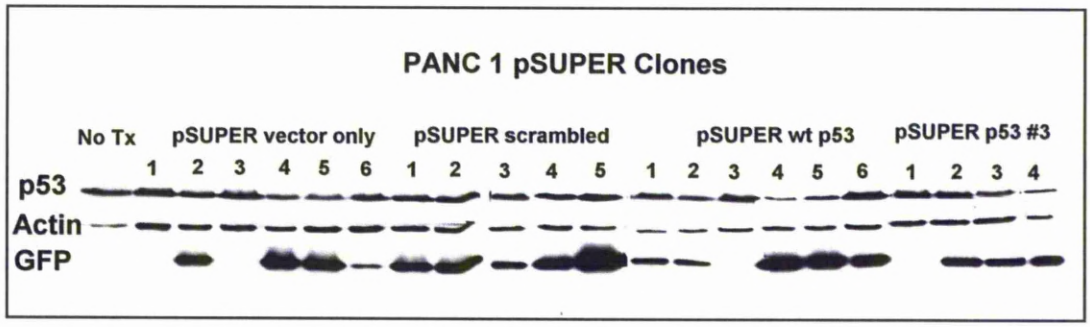



***Figure 5.3: p53 expression levels from the panel of pSUPER PANC 1 clones.***

*Cells were obtained, following neomycin antibiotic selection, from PANC1 clones that had undergone transfection with the pSUPER plasmid and its derivatives containing the scrambled, wild-type p53 and p53#3 mutant inserts (Section 2.8.2 – 2.8.8). 50µg/20µl of the harvested protein product was analysed by western blot using the the DO-1 (Ab 6) p53 primary antibody (Section 2.4).*

Figure 5.3 demonstrates that clones 2, 4 and 5 of the wild type expressing vector and clones three and four of the p53 mutant expressing vector appear to show a reduction in p53 protein expression. The shRNA sequence designed to target wild-type rather than mutant p53 protein does not appear to demonstrate specificity, with protein knock down levels comparable with the mutant specific sequence. The proportion of clones screened relative to those demonstrating a reduction in protein level, as well as the variability of EGFP expression, suggest that as predicted, inter clone

variability is likely to be a significant issue. Therefore, when picking colonies of colorectal cell lines, large number of clones would need to be screened to identify clones with the optimum p53 suppression.

## 5.5 EGFP expression in colorectal cells

Subsequently further clones were produced, using the same pSUPER / neomycin vector and shRNA insert derivatives as outlined in section 5.3, but using the SW620 colonic cancer cell line. Larger numbers of colonies were picked to establish clonal populations than in section 5.3, in order to accommodate for the potential variability in p53 suppression levels seen previously within the PANC 1 shRNA expressing clones. EGFP expression was initially assessed visually utilising UV fluorescence microscopy and then quantified alongside the effect on p53 protein expression by western blotting.

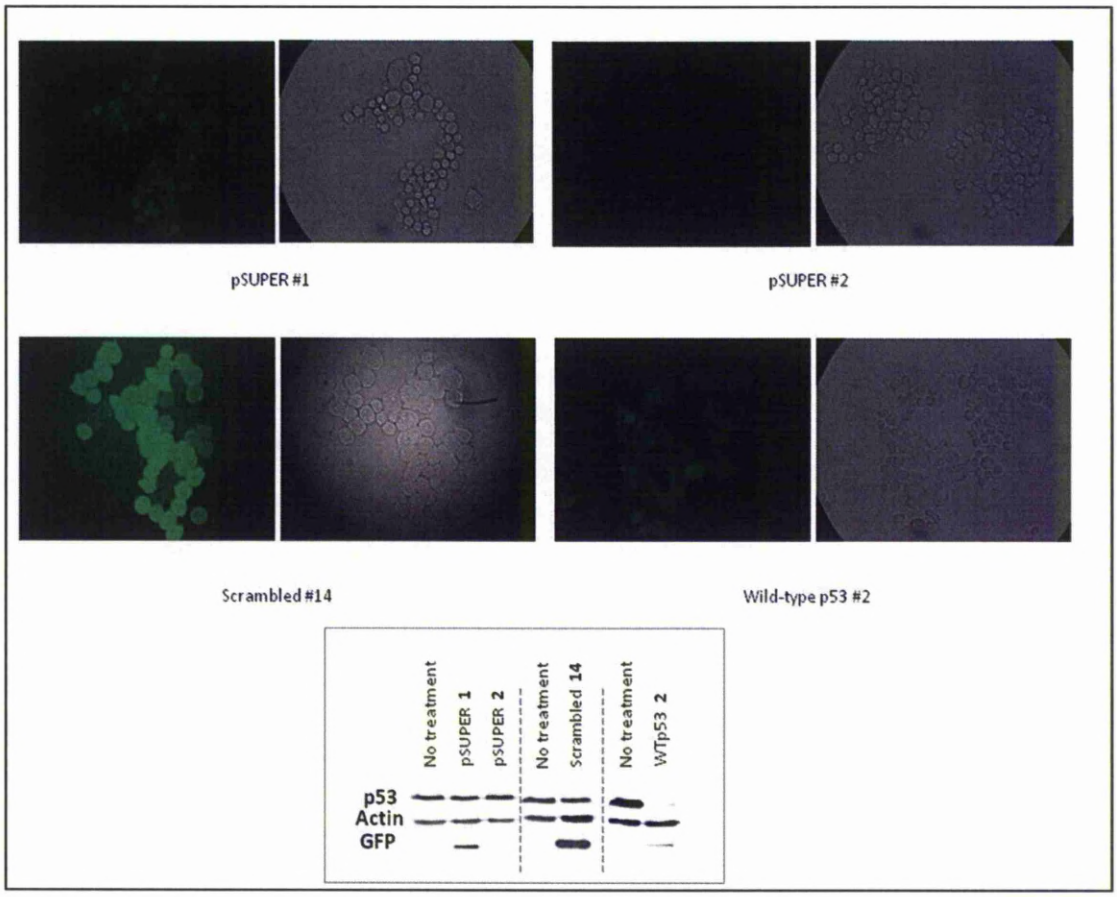



**Figure 5.4: Examples of EGFP expression variability in a sample of SW620 clones as determined by UV fluorescence microscopy and western blot analysis**

*Cells were harvested, plated, viewed and photographed as in the Figure 5.3 legend. The clonal populations displayed have been selected from the large number isolated and imaged in order to highlight the spectrum of EGFP expression witnessed. A western blot analysis of 50µg/20µl of the protein harvested from the lyastes of these cells is displayed concurrently. Due to the large number of clones processed and imaged, multiple blots were required, so each clonal population is displayed alongside a 'no treatment' control for that specific SDS Page gel (see section 2.4).*

The fluorescence photomicrographs shown in Figure 5.4 demonstrate that whilst some cell clones expressed EGFP protein and hence had undergone successful transfection with the pSUPER plasmid, other clones e.g. pSUPER #2, were not expressing EGFP detectably despite having successfully grown under neomycin selection. This could be accounted for by the development of neomycin resistance or could represent clones that have stably acquired the plasmid, but due to

rearrangement on integration, have lost the region or part thereof which encodes EGFP, or this may have become silenced e.g. epigenetically.

EGFP expression is seen to vary from clone to clone, for example it is much greater in scrambled clone #14 than in pSUPER clone #1, and the western blot reinforces this quantitative variability and correlates well with the visual impression from the photographs. There was also universal variability between levels of expression from neighbouring cells within a clonal population, i.e. the EGFP expression was not a uniform luminosity across the slide. In cell lines with no GFP demonstrable on western blot, there would often be a faint luminescent glow from just one or two cells seen on fluorescence microscopy, perhaps adding weight to mutation / silencing rather than antibiotic resistance as the reason for the clone survival.

Altogether GFP expression was detected by western blot in the following proportion of clones:

**Table 5.1: Proportion of SW620 clonal populations expressing GFP protein on western blot.**

| shRNA sequence insertion | GFP expression visible on western blot | Total number of clones studied for this shRNA insert |
|---|---|---|
| pSuper plasmid | 5 | 14 |
| Scrambled | 4 | 19 |
| R273H p53 mutant | 9 | 33 |
| Wild-type p53 | 19 | 30 |

All of these 97 clonal cell lines were subsequently analysed by western blot analysis to determine GFP and p53 protein levels.

## 5.6 Analysis of the pSUPER clonal populations generated in the SW620 colon cancer cell line.

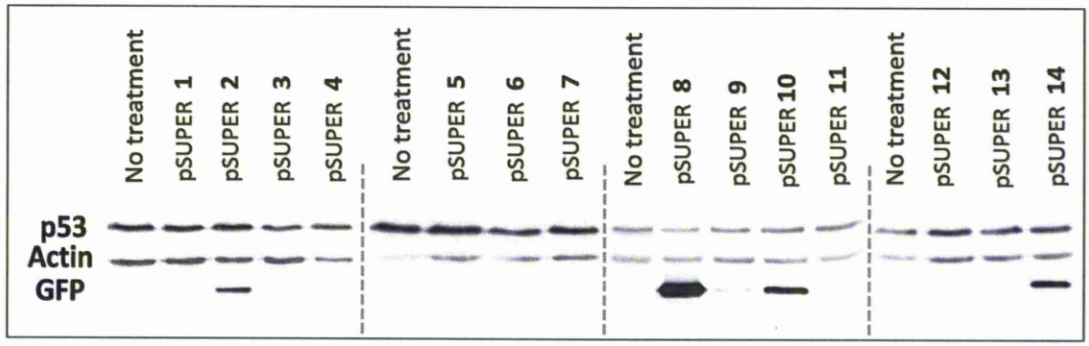



**Figure 5.5: SW620 pSUPER / neomycin vector only clones**

*(For Figures 5.5 to 5.9 the methodology of the pSUPER clones production is outlined in sections 2.8.2 to 2.8.8 and western blots were performed as detailed in section 2.4)*

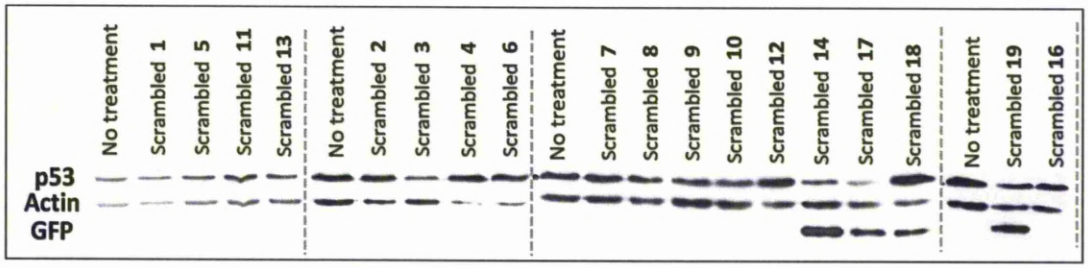



**Figure 5.6: SW620 scrambled shRNA expressing pSUPER clones**

In Figures 5.5 and 5.6 the pSUPER / GFP backbone only SW620 clones and the vector with the scrambled, and intended non-coding, shRNA sequence are analysed by western blot to assess their relative GFP and p53 protein expression. Ideally these negative control samples would show p53 levels comparable to the 'no treatment' parental cell line, thereby demonstrating no effect of the cloning process on p53

levels and that any reduction in p53 levels in the p53-targeted shRNA cell lines could therefore be considered specific. Due to the number of clonal cell lines, multiple blots were required to examine this question, with each group of samples on a single blot being compared with a no treatment control run on the same gel, to act as a reference.

p53 levels in the pSUPER backbone clones seemed generally comparable with the no treatment control, though clones 3 and 4 and to a lesser degree clone 6, did show a slight reduction in p53 expression when compared with the parent cell line as demonstrated in Figure 5.5. Interestingly, only 5 clones displayed any detectable GFP expression and the p53 levels were comparable with the parental cell line in these clones, hence providing useful control cell lines for our subsequent experiments.

In Figure 5.6 it is shown that the scrambled clones showed slightly more variability in p53 protein expression, with p53 expression reduced in clones 3, 14 and 17 and increased in clone 18 when compared with the SW620 parental cell line. Of the 19 clones, only four showed any GFP expression and unfortunately three of these appeared to also have altered p53 protein levels. This raises the possibility that the shRNA scrambled sequence could be having an unexpected (for example off-target) effect on the p53 pathway (though this had not been observed previously with the siRNA sequence which had been used routinely in our laboratory). Alternatively further mutations may have occurred elsewhere in the p53 pathway within these clonal populations, or the selection process itself may have caused an alteration in mutant p53 expression by up or down regulation of the intrinsic protein levels. Regardless of aetiology of the altered p53 levels, this would need to be considered when selecting appropriate control clone cell lines for future experiments.

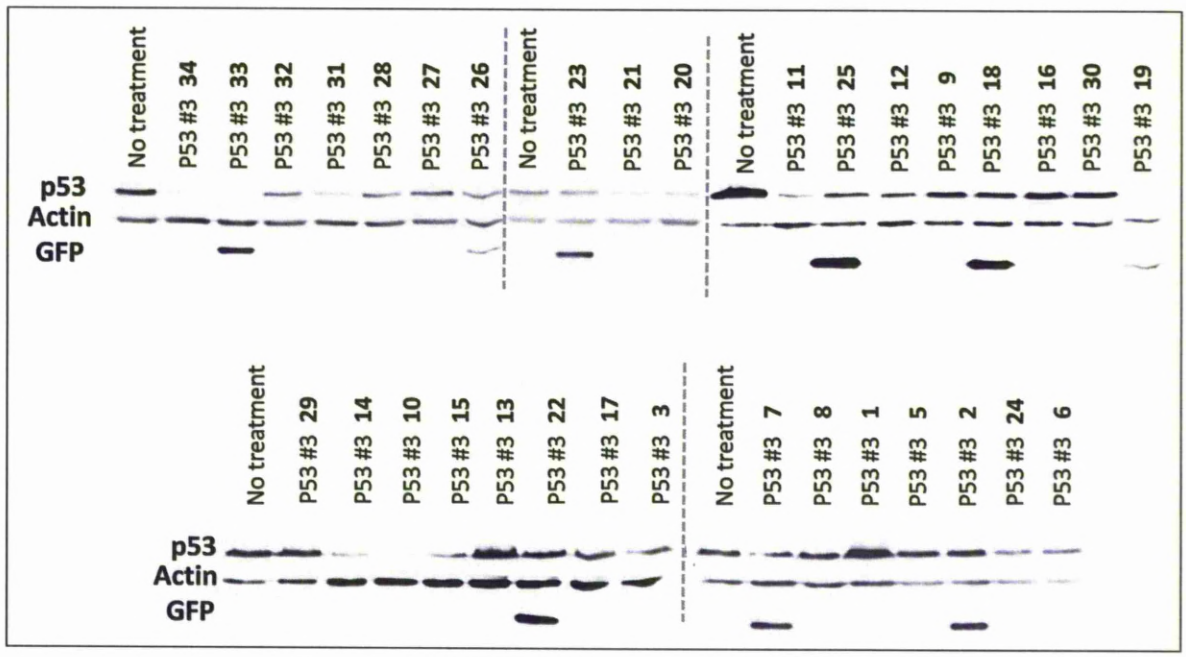



**Figure 5.7: SW620 R273H mutant p53 shRNA expressing pSUPER clones**

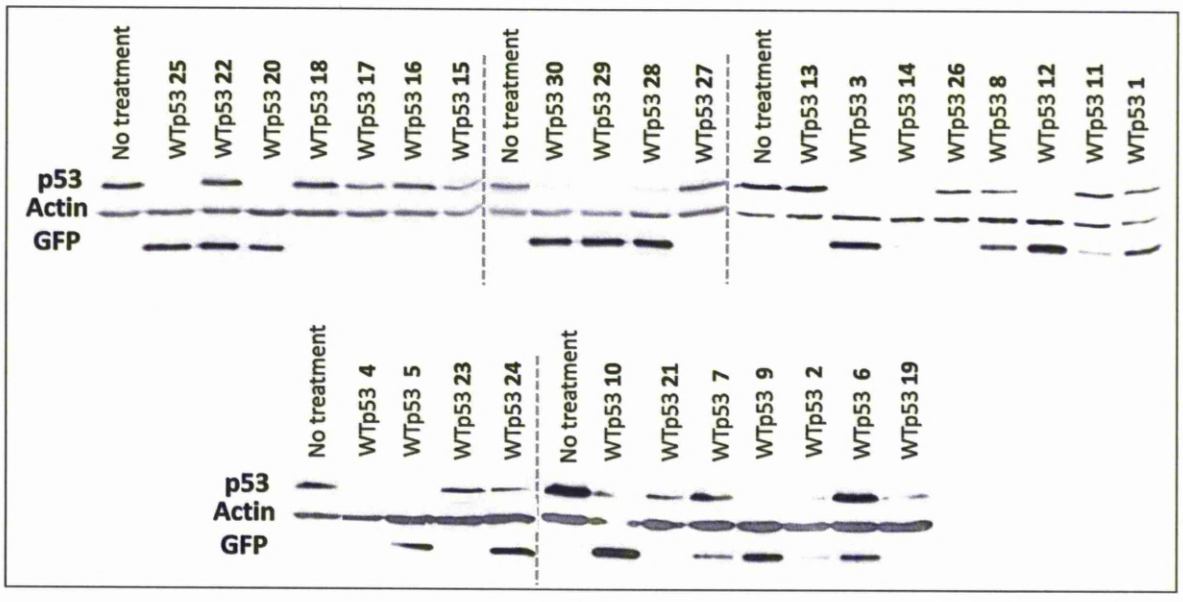



**Figure 5.8: SW620 wild-type p53 shRNA expressing pSUPER clones**

Figure 5.7 demonstrates the p53 protein levels within the SW620 clones which contain the pSUPER vector expressing the shRNA sequence specifically targeted at

the R273H mutation. In order to allow comparison of degree of p53 suppression between clones, the protein reduction was visually compared with the no treatment control group for each western blot and hence broadly categorised as shown in table 5.2.

p53 protein reductions of >75% (based on visual categorisation) are seen in some clones though others show no apparent change in protein expression levels. Once again GFP expression was not evident in all of the clones, but reduction in p53 steady-state levels was demonstrated independently of this, suggesting successful plasmid transfection, with lack of expression of the GFP component, rather than antibiotic resistance as the underlying mechanism.

From this data the following summary of p53 knock down within the R273H shRNA clones can be produced:

- 5 of the 33 clones showed a >75 % reduction in p53 expression
- 25 clones in total showed a detectable reduction in p53 protein levels
- GFP expression was detectable in 9 of the 33 clones but the level of this was not correlated with p53 expression levels.

Figure 5.8 demonstrates the degree of p53 suppression in the SW620 clones that underwent transfection with the pSUPER vector expressing the wild-type p53 shRNA. There does not appear to be any demonstrable specificity of the shRNA sequences, with the wild-type sequence showing equal, if not greater, p53 knockdown than the mutant specific sequence. This observation is not in accord with previous studies, which showed a single base pair alteration is sufficient for specificity of the shRNA [244]. I think more detailed studies and quantification, for

example with densitometry, would be required to interpret this properly and to state whether these results truly contradict the previously published work.

A summary of the p53 suppression with the wild-type targeted shRNA sequence is summarised below:

- 12 of the 30 clones showed a >75% reduction in p53 expression
- 22 of the clones showed a detectable reduction in p53 protein levels
- GFP expression was seen to some extent in 20 of the 33 clones but again this showed no correlation with the degree of p53 knock down.

***Table 5.2: Summary of p53 and GFP protein levels in the SW620 pSUPER clones***

| Clone | P53 expression | GFP expression | Clone | P53 expression | GFP expression | Clone | P53 expression | GFP expression |
|---|---|---|---|---|---|---|---|---|
| pSUPER 1 | Same | None | P53#3 1 | Increased | None | WTp53 1 | 50 – 75% reduction | Moderate |
| pSUPER 2 | Same | Moderate | P53#3 2 | Same | Moderate | WTp53 2 | >75% reduction | Low |
| pSUPER 3 | <50% reduction | None | P53#3 3 | <50% reduction | None | WTp53 3 | >75% reduction | High |
| pSUPER 4 | <50% reduction | None | P53#3 5 | Same | None | WTp53 4 | >75% reduction | None |
| pSUPER 5 | Same | None | P53#3 6 | 50 – 75% reduction | None | WTp53 5 | >75% reduction | Moderate |
| pSUPER 6 | <50% reduction | None | P53#3 7 | 50 – 75% reduction | Moderate | WTp53 6 | Same | Moderate |
| pSUPER 7 | Same | None | P53#3 8 | Same | None | WTp53 7 | <50% reduction | Moderate |
| pSUPER 8 | Same | High | P53#3 9 | <50% reduction | None | WTp53 8 | 50 – 75% reduction | Moderate |
| pSUPER 9 | Same | Low | P53#3 10 | >75% reduction | None | WTp53 9 | >75% reduction | High |
| pSUPER 10 | Same | Moderate | P53#3 11 | >75% reduction | None | WTp53 10 | 50 – 75% reduction | High |
| pSUPER 11 | Same | None | P53#3 12 | 50 – 75% reduction | None | WTp53 11 | Same | Low |
| pSUPER 12 | Same | None | P53#3 13 | Increased | None | WTp53 12 | >75% reduction | High |
| pSUPER 13 | Same | None | P53#3 14 | 50 – 75% reduction | None | WTp53 13 | Same | None |
| pSUPER 14 | Same | Moderate | P53#3 15 | 50 – 75% reduction | None | WTp53 14 | >75% reduction | Low |
| Scram 1 | Same | None | P53#3 16 | <50% reduction | None | WTp53 15 | <50% reduction | None |
| Scram 2 | Same | None | P53#3 17 | <50% reduction | None | WTp53 16 | Same | None |
| Scram 3 | <50% reduction | None | P53#3 18 | <50% reduction | High | WTp53 17 | <50% reduction | None |
| Scram 4 | Same | None | P53#3 19 | >75% reduction | Low | WTp53 18 | Same | None |
| Scram 5 | Same | None | P53#3 20 | 50 – 75% reduction | None | WTp53 19 | 50 – 75% reduction | None |
| Scram 6 | Same | None | P53#3 21 | 50 – 75% reduction | None | WTp53 20 | >75% reduction | High |
| Scram 7 | Same | None | P53#3 22 | Same | High | WTp53 21 | 50 – 75% reduction | Low |
| Scram 8 | Same | None | P53#3 23 | Same | Moderate | WTp53 22 | Same | High |
| Scram 9 | Same | None | P53#3 24 | 50 – 75% reduction | None | WTp53 23 | Same | None |
| Scram 10 | Same | None | P53#3 25 | 50 – 75% reduction | High | WTp53 24 | <50% reduction | High |
| Scram 11 | Same | None | P53#3 26 | <50% reduction | Low | WTp53 25 | >75% reduction | High |
| Scram 12 | Same | None | P53#3 27 | <50% reduction | None | WTp53 26 | <50% reduction | None |
| Scram 13 | Same | None | P53# 28 | <50% reduction | None | WTp53 27 | Same | None |
| Scram 14 | <50% reduction | High | P53#3 29 | Same | None | WTp53 28 | >75% reduction | High |
| Scram 15 | Same | None | P53#3 30 | <50% reduction | None | WTp53 29 | >75% reduction | High |
| Scram 16 | Same | None | P53#3 31 | 50 – 75% reduction | None | WTp53 30 | >75% reduction | High |
| Scram 17 | <50% reduction | Moderate | P53#3 32 | <50% reduction | None | | | |
| Scram 18 | Increased | Moderate | P53#3 33 | >75% reduction | Moderate | | | |
| Scram19 | Same | High | #3 34 | >75% red | None | | | |

## 5.7 Analysis of the pSUPER clonal populations generated in the SW480 colon cancer cell line.

Subsequently pSUPER plasmid containing clones were also created in the SW480 colon cell line to see if any witnessed effects of stable p53 knock down would be consistent across two different cell lines. Due to time constraints, transfection was undertaken only with the wild-type p53 shRNA plasmid, alongside the previously described pSUPER and scrambled sequence controls (section 2.8.2).

The clonal populations produced from the transfection of this second colorectal cell line with the pSUPER vector and shRNA expressing derivatives are demonstrated below in figure 5.9.

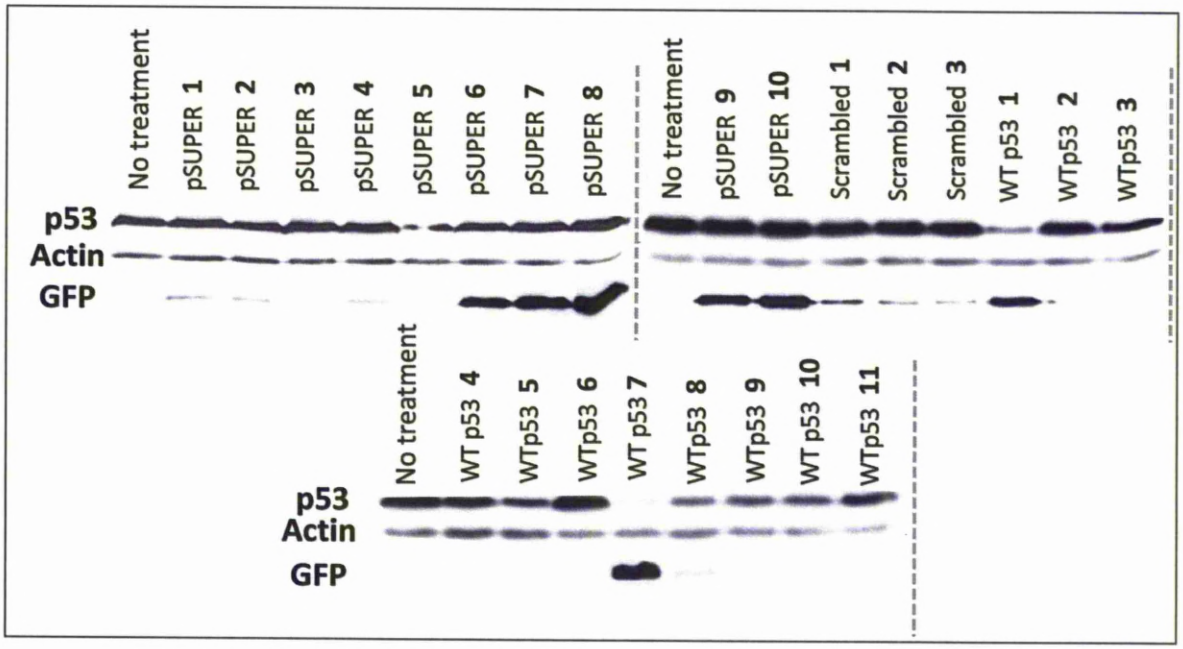



**Figure 5.9: SW480 clones containing pSUPER / neomycin vector only, scrambled and wild-type p53 shRNA sequences**

Figure 5.9 shows steady-state p53 protein levels in all of the state SW480 cell line pSUPER and scrambled control clones with a large proportion of these also demonstrating GFP expression. Seven of the eleven wild-type clones demonstrate a reduction in p53 protein levels, with two of these being >75%. As the two clones with the greatest reduction in p53 levels also showed high levels of GFP expression, these would be good candidates for further investigation into the impact of this p53 suppression, since the changes in p53 expression levels are more likely to due to successful transfection with the shRNA plasmid, rather than intrinsic protein level fluctuations, if the GFP component of the plasmid is functional.

Having established this array of stable clonal populations with suppressed p53 levels in two mutant p53 expressing colorectal cell lines, these could then be studied further to evaluate the impact of this protein suppression on cell proliferation rates and response to chemotherapeutic agents.

# Chapter 6. Studies of the impact of mutant p53 suppression on cell proliferation in the SW620 colon cancer cell line

My hypothesis is that colorectal cancer cells with expression of high levels of mutant p53 protein may display less malignant potential if the level of mutant p53 expression were reduced, as discussed previously in section 1.7. One potential mechanism driving cancer disease progression is an increased rate of cell division, in comparison with the non-cancerous cells, allowing for tumour growth. However, it must also be considered that cancer cells may not divide more rapidly, but rather that they undergo a reduced rate of apoptosis compared with the parent cell, hence leading to tumour progression.

An experiment was designed to try and ascertain if the rate of cell division was altered by suppression of the mutant p53 protein levels in the p53 mutant SW620 colon cancer cell line. A selection of the clonal populations containing the pSUPER shRNA plasmid, which had previously demonstrated a reduction of the levels of mutant p53 expression on western blot in the previous chapter (see section 5.6), were compared with the initial parent line to determine whether any alteration in cell division rates could be linked with the reduction in mutant p53 protein expression. This was studied by counting the total number of cells present in standard volume tissue culture and assessing the rate of increase over a measured time period and observing any differences between the mutant p53 clonal populations when compared with the parent cell line.

## 6.1 Selection of the clonal populations for proliferation studies

In section 5.6 the production of an array of SW620 clonal populations containing the pSUPER plasmid, with shRNA expressing sequences targeted at mutant and wild-type p53 sequences, was discussed. The production of clones containing the empty pSUPER/neomycin vector and the creation of clones expressing a non-targeting, so called scrambled, sequence was also outlined. In this experiment a pSUPER SW620 cell line containing the pSUPER plasmid with no insert and a representative clone with a scrambled shRNA sequence were selected for analysis alongside the parental cell line to act as controls. These were utilised as controls for the specificity of the shRNA mediated p53 suppression; since all of the clones contain the plasmid, this suggests that any difference in proliferation rates are more likely to be attributable to whether they harbour a p53 specific shRNA sequence, rather than a result of the cloning process. Hence, these were examined alongside the p53 shRNA expressing clones to determine whether stable reduction in p53 by shRNA altered the rate of cell proliferation.

As shown in Figure 6.1, the level of p53 expression in the empty vector and scrambled shRNA clones chosen were comparable to the parental cell line (or some such statement) From the collection of clones containing the empty pSUPER vector, one with a moderate level of GFP expression was selected for this experiment. As no shRNA scrambled sequence clones analysed to this point had demonstrated any GFP expression detectable by western blotting, the first clone obtained was selected to be studied. As discussed in section 5.6, mutant p53 protein expression was visualised in some of the clonal populations transfected with both the wild type and the R273H mutant targeted shRNA sequences. Initially one mutant p53 shRNA population with

moderate suppression (50 – 75 % reduction, p53#3 25) and one with a more effective suppression of the mutant protein using the wild-type p53 sequence (>75% reduction, WTp53 3) were selected for cell proliferation analysis. These particular cell lines were chosen since they had retained their GFP expression as well as demonstrating the suppression of the mutant p53 protein levels.

The relative p53 and GFP expression profiles of the clones selected for this proliferation experiment are demonstrated on western blot in Figure 6.1:

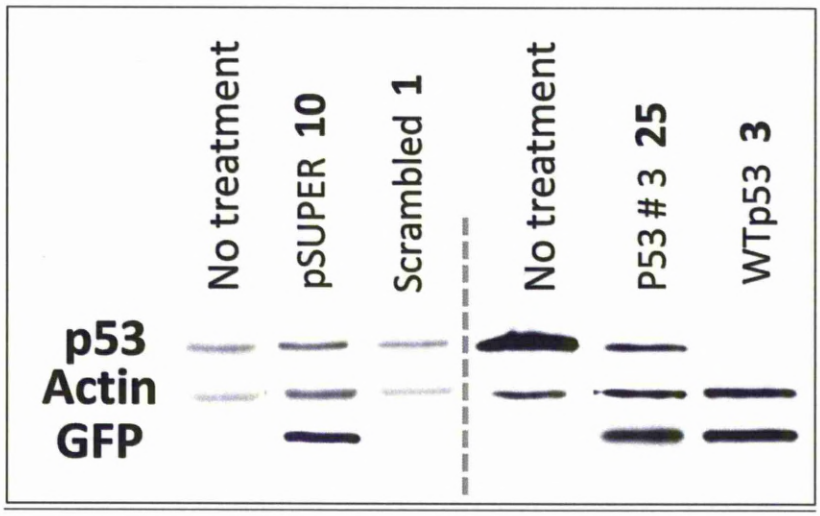



***Figure 6.1: Western blot analysis of the pSUPER clones for the initial proliferation rate experiment***

*(Summarised from previous data presented in Figures 5.5 to 5.8)*

## 6.2 Proliferation analysis of SW620 cell clones expressing variable levels of mutant p53.

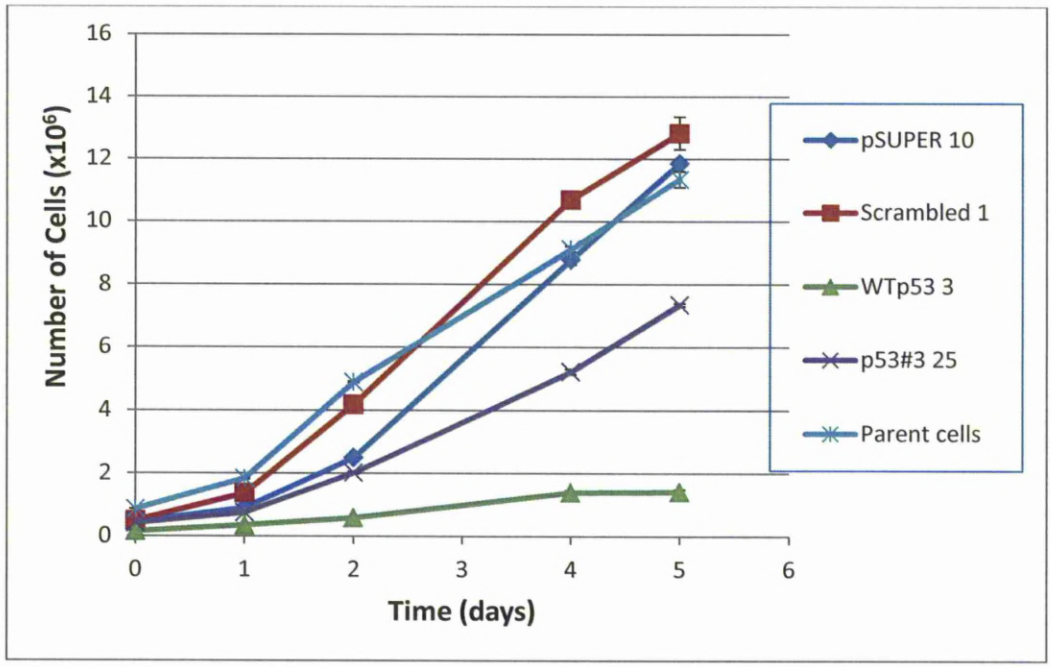



***Figure 6.2: Proliferation analysis of SW620 cell clones expressing variable levels of mutant p53.***

*Cells were plated onto 6 well plates (area 9.5cm$^2$ / well) in their usual growth media aiming for a confluence 24 hours later (Day 0) of approximately 10%. Cells were harvested every 24 hours and total cell count per well ascertained using a coulter counter (Beckman Coulter – Z series, Beckman Coulter Inc.) The coulter counter was maintained, flushed and calibrated as per manufacturer's instructions. Cells were harvested in the smallest volume needed for adequate trypsinisation. 0.5ml of these harvested cells formed the metered volume for the Coulter analysis. This cell suspension was combined with 10 ml of isoton II diluent (Beckman Coulter inc.) to fill the accuvette (Beckman Coulter inc.) and using a 100µm aperture tube all particles between 8 and 20 µm were filtered and counted. This produced a value expressed as [cells / ml] which was then multiplied by the appropriate total initial harvested volume (0.5ml – 5ml), as necessary. The average of three wells per condition was plotted with error bars displaying the SEM.*

In figure 6.2 the rate of cell proliferation appears to be comparable in the three control lines (parental cells, scrambled and empty pSUPER vector) but there appears to be a significant reduction in the growth rates of both of the populations in which the mutant p53 protein expression is suppressed. This appears to be proportional to the degree of p53 suppression, with the moderately suppressed p53#3 25 clone

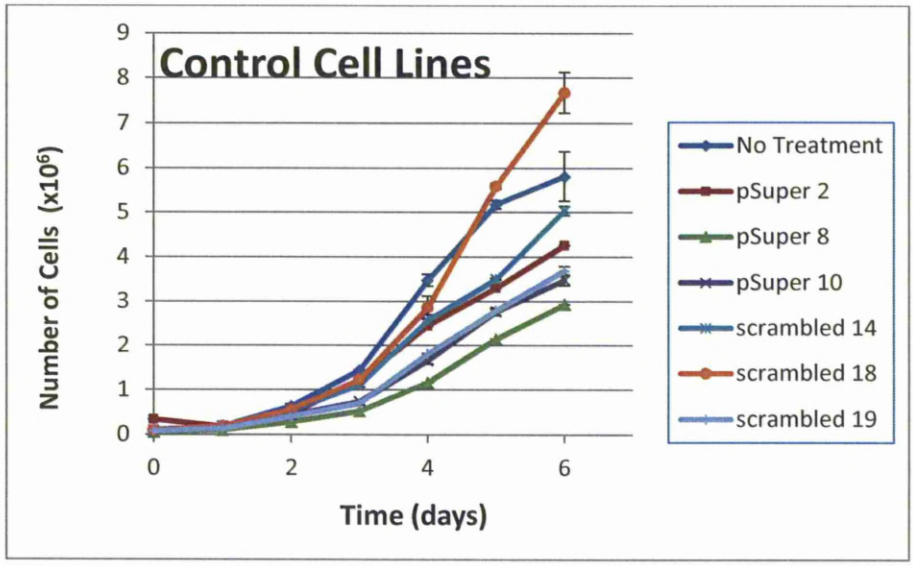
showing a moderate restriction in cell proliferation with a greater reduction noted in the WTp53 3 cell line which had a more marked p53 suppression.

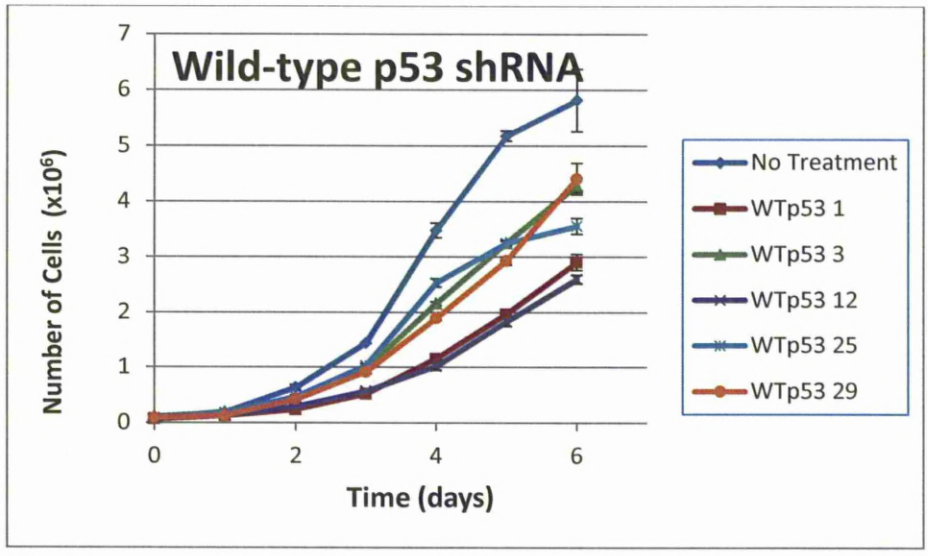
To determine whether the observation in Figure 6.2 held true in additional clones of sw620 cells, the experiment was repeated with a larger selection of the shRNA pSUPER mutant p53 supressed cell populations. A selection of clones containing the pSUPER p53 wild-type and mutant targeted sequences were chosen to reflect a range of mutant p53 suppression levels as summarised in Table 5.2 (see also Figures 5.5 – 5.8 for western blots). Several of the pSUPER empty vector and scrambled sequence clones which showed GFP expression, but minimal alteration in p53 expression levels, were also selected as controls, as discussed in section 6.1.

***Table 6.1: Summary of relative p53 and GFP protein levels in the selected SW620 pSUPER cell lines***

*(Original western blot data presented in Figures 5.5 – 5.8)*

| Cell Line | p53 expression | GFP expression |
|---|---|---|
| pSuper 2 | *As per parent cell line* | *Moderate* |
| pSuper 8 | *As per parent cell line* | *High* |
| pSuper 10 | *As per parent cell line* | *Moderate* |
| Scrambled 14 | *Slight reduction* | *High* |
| Scrambled 18 | *Slight increase* | *Moderate* |
| Scrambled 19 | *As per parent cell line* | *High* |
| WTp53 1 | *50 – 75% reduction* | *Moderate* |
| WTp53 3 | *>75% reduction* | *High* |
| WTp53 12 | *>75% reduction* | *High* |
| WT p53 25 | *>75% reduction* | *High* |
| WTp53 29 | *>75% reduction* | *High* |
| p53#3 19 | *>75% reduction* | *Low* |
| p53#3 22 | *As per parent cell line* | *High* |
| p53#3 25 | *50 – 75% reduction* | *High* |
| p53#3 33 | *>75% reduction* | *Moderate* |



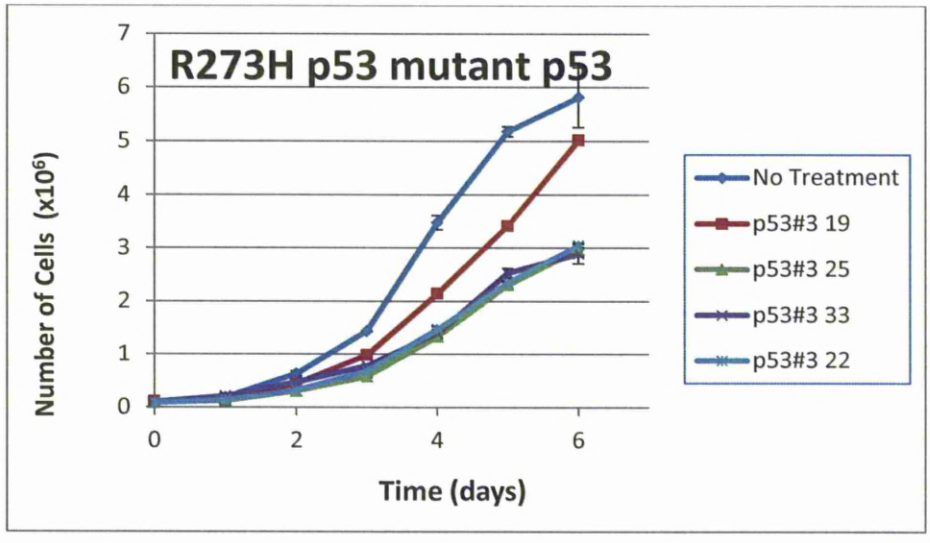




*Figure 6.3: Further proliferation experiments with an array of SW620 pSUPER cell lines*

## 6.3 Calculation of Proliferation Rate using a Logarithmic Scale, Slope and Pearson's Correlation Co-efficient.

These graphs demonstrate an exponential increase in cell number for any given time increment, with some of the curves flattening out as cell numbers increase, presumably as nutrients and space become an issue. We might expect the proliferation curves to follow a logarithmic rate of expansion based on binary cell division until achieving a density where further expansion is limited by competition for resources. Such logarithmic distributions make any visual comparison of proliferation rates difficult since small discrepancies in plating densities will make large differences to daily cell numbers, whereas the underlying rate of proliferation may be comparable. Therefore to allow a more accurate comparison of the cell populations I calculated the $\log_{10}$ value of the cell numbers, converting the exponential graphs into linear ones and hence allowing rate of growth to be calculated by ascertaining the slope of the line (b). Where b = [Log10(cell number)]/Day .The slope was ascertained by establishing a linear regression using the equation:

$$b = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2}$$

where x and y are the sample means AVERAGE(known_x's) and AVERAGE (known_y's).

To confirm that this was appropriate, the extent of a linear relationship between $\log_{10}$ (cell number) and time for each population was confirmed by calculating Pearson's correlation co-efficient as follows:

$$r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

where x and y are the sample means AVERAGE(array1) and AVERAGE(array2).

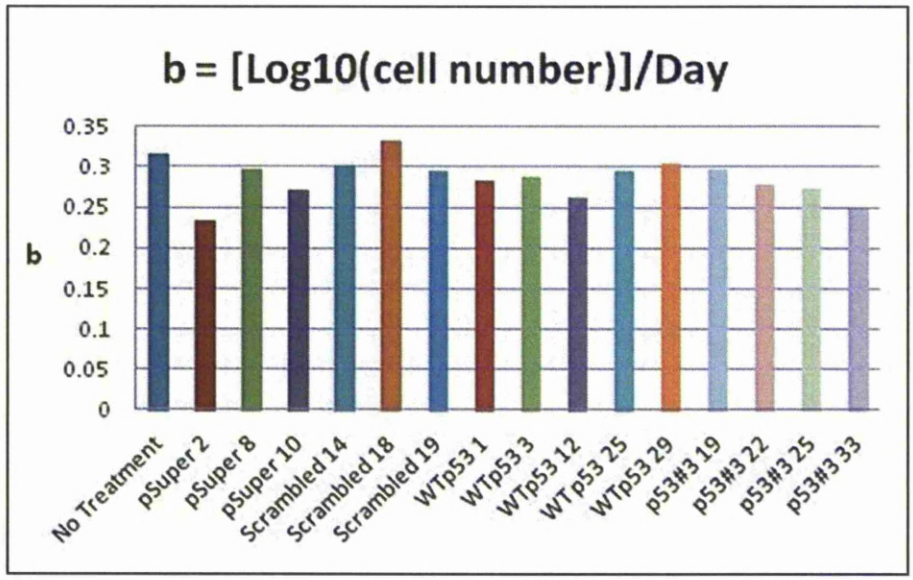



***Figure 6.4: Histogram showing Proliferation Rates for an array of SW620 pSUPER Clones***

***Table 6.2: Pearson's Correlation co-efficient for the Logarithmic Proliferation Linear Regression***

| Cell Line | Pearson's |
|---|---|
| No Treatment | 0.98 |
| pSuper 2 | 0.95 |
| pSuper 8 | 0.99 |
| pSuper 10 | 0.99 |
| Scrambled 14 | 0.99 |
| Scrambled 18 | 0.99 |
| Scrambled 19 | 0.99 |
| WTp53 1 | 1.00 |
| WTp53 3 | 0.99 |
| WTp53 12 | 1.00 |
| WT p53 25 | 0.97 |
| WTp53 29 | 0.99 |
| p53#3 19 | 0.99 |
| p53#3 22 | 1.00 |
| p53#3 25 | 0.99 |
| p53#3 33 | 0.99 |

All of the Pearson's correlation co-efficients are $\geq 0.95$ demonstrating a good linear regression and therefore confirming the appropriateness of comparison of proliferation rates by this method.

There does appear to be variability in the rates of cell proliferation between the pSUPER clones and the native cell line but whilst the rate of cell proliferation appears lower in all of the clones with suppressed p53, there are also clones containing the empty backbone vector and the scrambled sequence that demonstrate a reduction in the rate of cell proliferation. This could suggest that the process of clonal selection is responsible for alterations in cell proliferation rather than a specific effect of the reduction of mutant p53 protein expression. The scrambled 18 clone is an exception to this finding, in that there appears to be an increase in the rate of cell proliferation in this population when compared with the parent cell line. Western blot analysis of this cell line also displayed a slight increase in p53 protein levels and it may be that a higher level of mutant p53 expression has been selected for within this clonal cell population.

Therefore in summary, these experiments provide no evidence for a role for mutant p53 expression in cell proliferation in the studied SW620 clones.

# Chapter 7. Studies of the impact of mutant p53 suppression on the response to chemotherapeutic agents in the SW620 colon cancer cell line

## 7.1 Hypothesis

The literature suggests that over-expression of at least some forms of mutant p53, may directly enhance the resistance of tumour cells to anti-cancer agents and therefore that p53 gain-of function might contribute to failure of chemotherapy [235], see section 1.1.25. The reduction of mutant p53 expression in the p53 knock down pSUPER clones may render them more susceptible to the effects of chemotherapy and therefore rates of cell proliferation may be restricted by lower drug concentrations. The effects of two chemotherapeutic agents 5 fluorouracil (5FU) and Oxaliplatin were therefore studied in these cells; since these drugs are commonly used in the treatment of primary and metastatic colon cancer (see 1.1.11).

## 7.2 Determination of $IC_{50}$ for 5-fluorouracil and Oxaliplatin

$IC_{50}$ (The half maximal inhibitory concentration) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug is needed to inhibit a given biological process, in this case cell proliferation, by 50 percent, at a specific time point. Initially the proliferation curve of the parent SW620 cell line was utilised, to calculate the $IC_{50}$ for exposure to both chemotherapeutic drugs within this population. This would allow the determination of an appropriate range of chemotherapy agent concentrations to apply to the mutant p53 suppressed pSUPER clones, in order to try and calculate their $IC_{50}$ in the subsequent experiments.

Following a review of previous similar studies with the SW620 cells and these chemotherapeutic agents, concentrations of [1-20μM] for 5FU and [0.25-10μM] for oxaliplatin were tested to establish the $IC_{50}$ for the parent population on cells harvested at 72 hours post exposure.

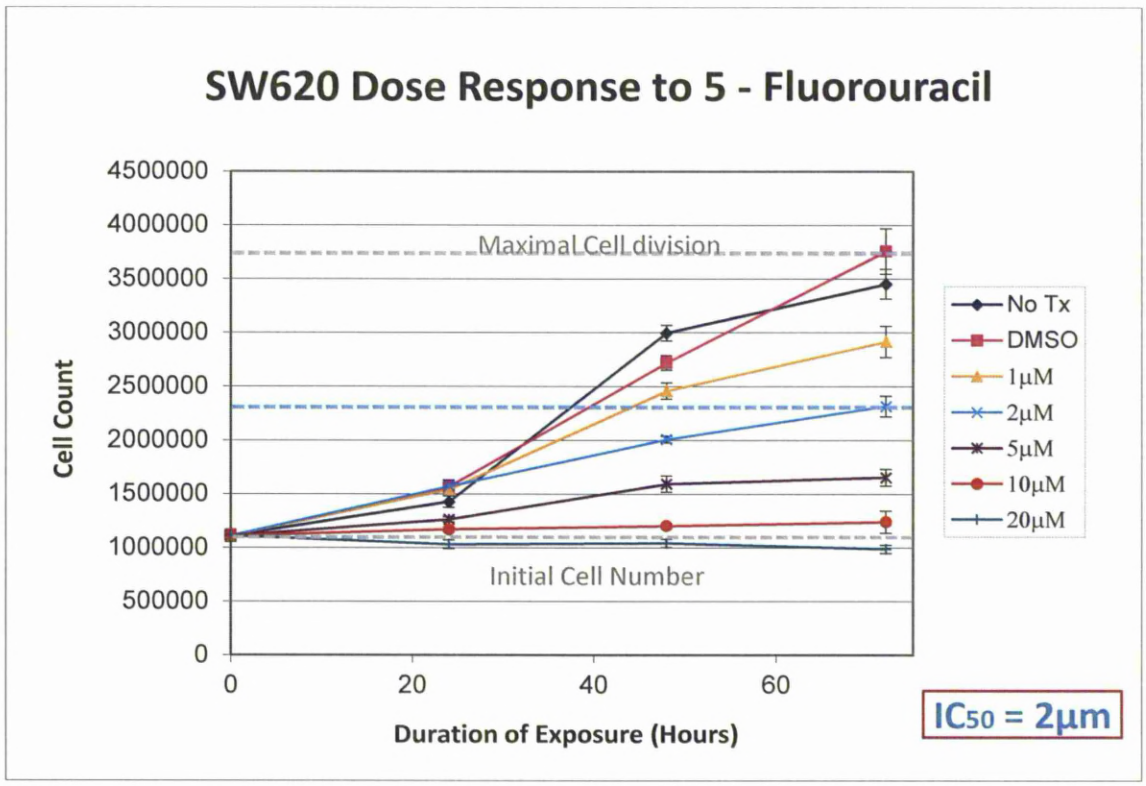



***Figure 7.1: Dose response curves for SW620 cells treated with 5 – Fluorouracil***

*5-fluorouracil (5-FU) (Sigma, $C_4H_3FN_2O_2$, MW = 130.8g) was dissolved in DMSO (0.065g /ml) [0.5M] stock and stored at 4°C. This was further diluted to [10mM] in DMSO and added to the appropriate tissue culture media volume (2ml / well) to obtain final working concentrations of 1 to 20μM 5-fluorouracil OR 5-FU. In order to try and avoid any adverse effects from DMSO toxicity, the volume of DMSO / 5-FU added to the media was kept at a minimum (0.02%). Cells were seeded in 6 well plates aiming for starting confluencies of 10-20% 24 hours later. Tissue culture media was removed and media containing the chemotherapeutic agents applied and left on the cells for the duration of the experiment. Cells were harvested and counted at 0, 24, 48 and 72 hours using the Coulter analyser and cell proliferation graphed and analysed using the methods discussed in section 2.9.*

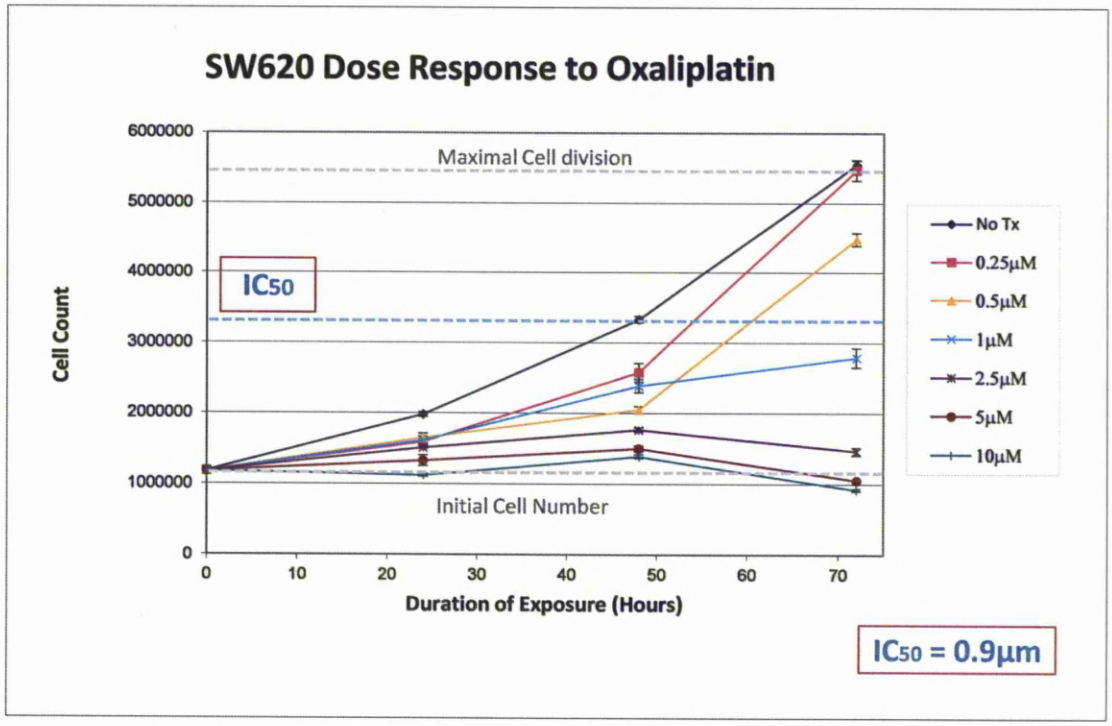



***Figure 7.2: Dose response curves for SW620 cells treated with Oxaliplatin***

*Oxaliplatin (LC Laboratories, $C_8H_{14}N_2O_4Pt$, MW = 397.29g) was dissolved in $H_2O$ (0.4g/ml) [1M] stock and stored at 4°C. This was diluted in water to [10mM] and added to the media to obtain [Oxaliplatin] 0.25μm – 10μm. The method was otherwise as stated in Figure 7.1.*

Figures 7.1 and 7.2 demonstrate a fairly clear dose : response relationship across the range of chemotherapy concentrations for both agents, with cell numbers suppressed proportionally by the increasing drug levels. This allows calculation of the $IC_{50}$ as follows

Maximal Cell Number = The Highest cell count at 72 hours.

Initial Cell Number = The mean cell count at 0 hours.

$IC_{50}$ = The concentration of chemotherapeutic drug needed to produce a cell count of:

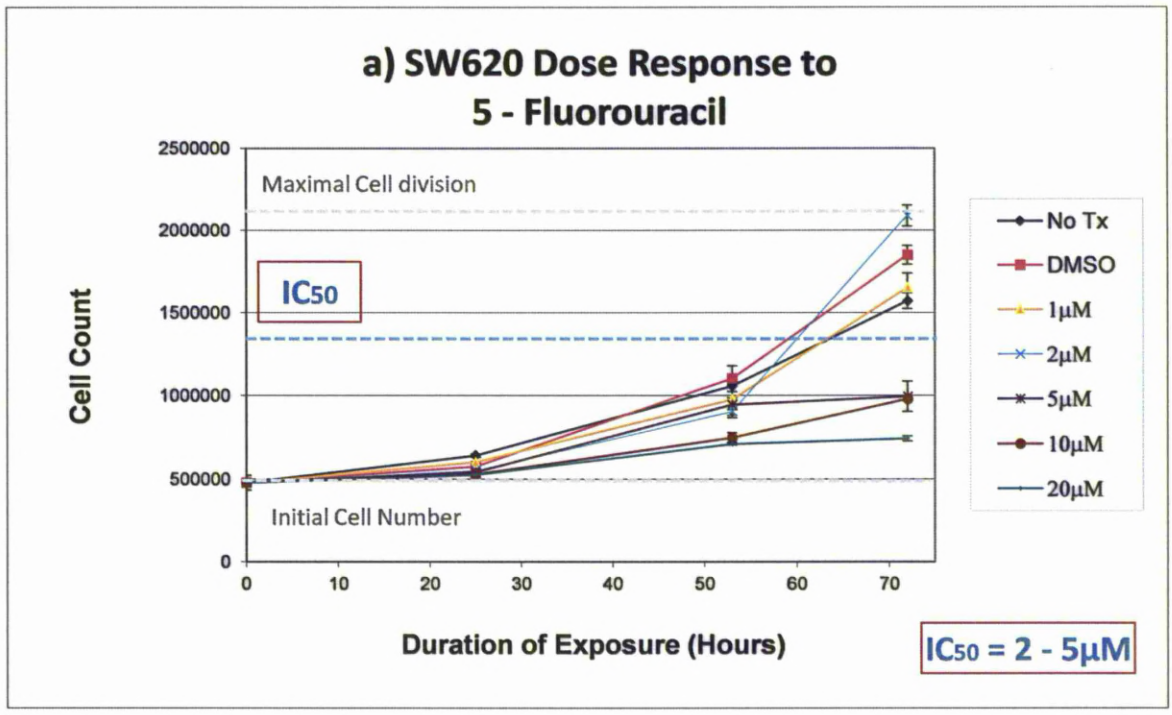
(Maximal Cell Number – Initial Cell Number)/2 + Initial Cell Number

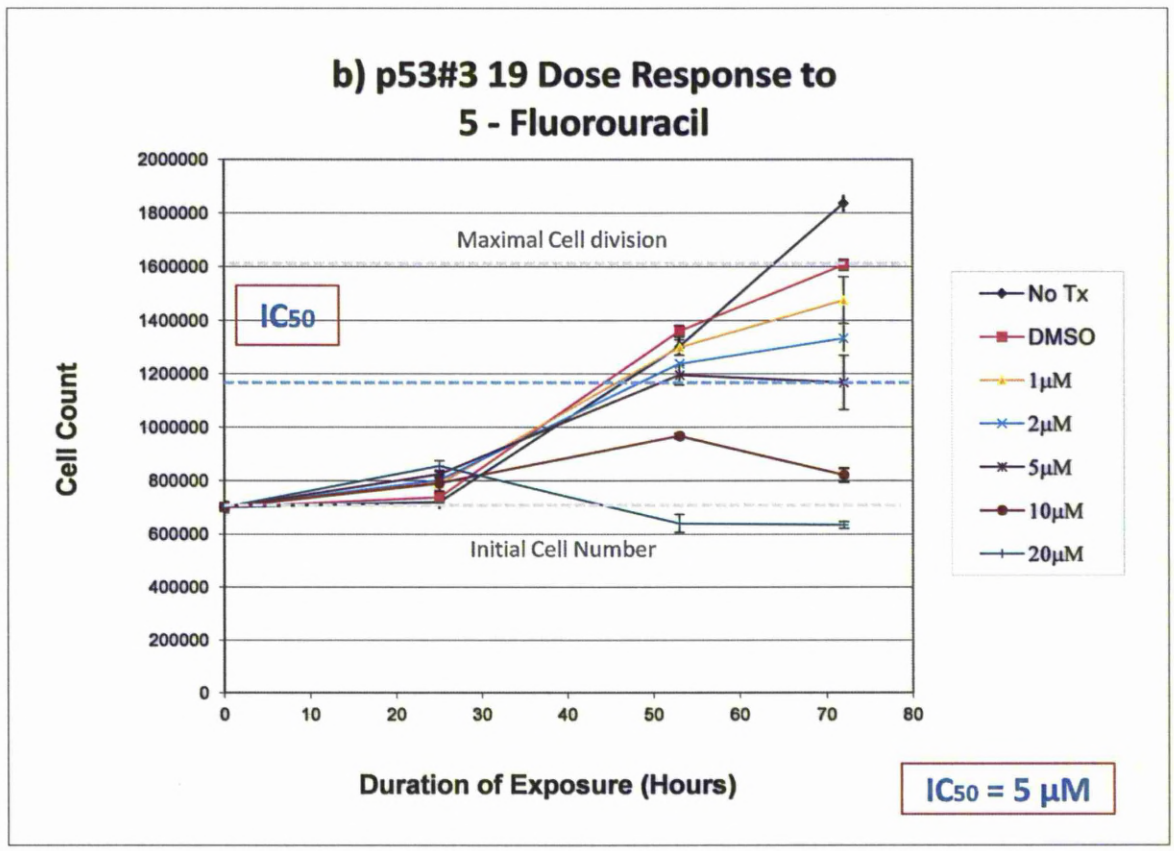
Therefore the IC50 for 5FU = 2μm and for oxaliplatin = 0.9μm in our parent SW620 cell line.

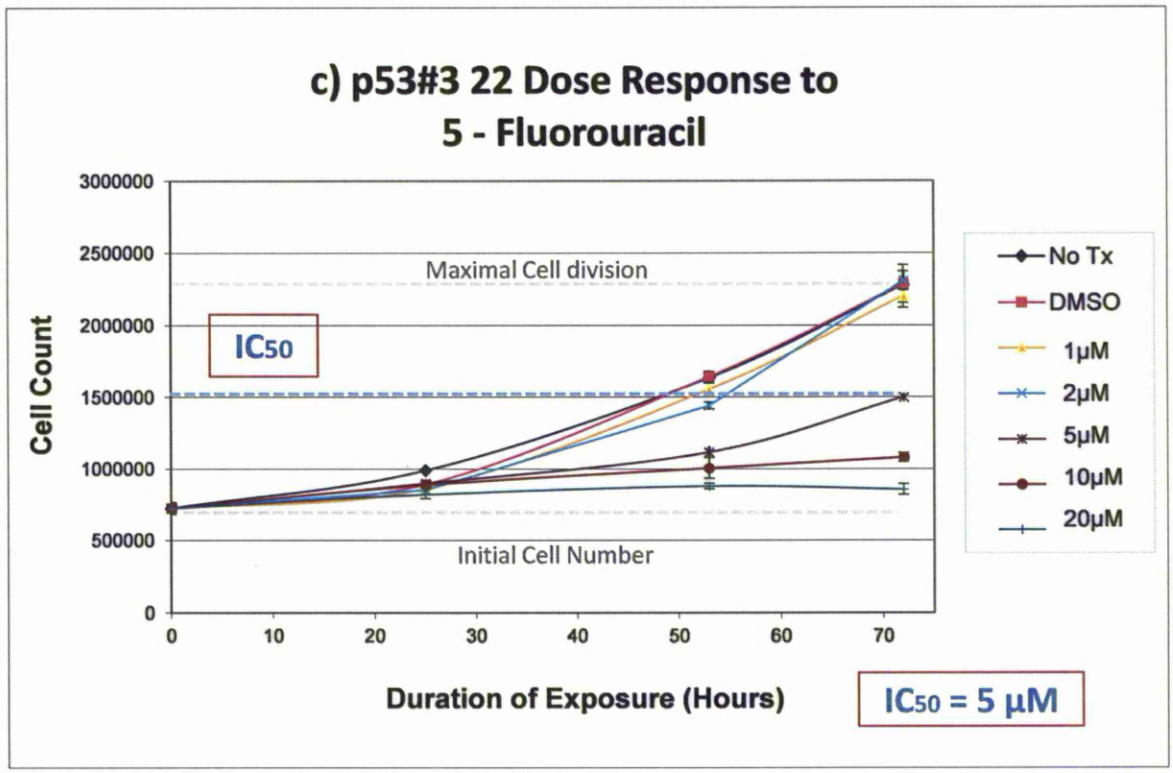
In the 5-FU experiments, the Maximal Cell Number was deemed to be that achieved with the DMSO treatment, to negate for any cytotoxicty due to this solvent rather than the chemotherapeutic drug being tested. This was unnecessary with oxalaplatin since the solvent is water.

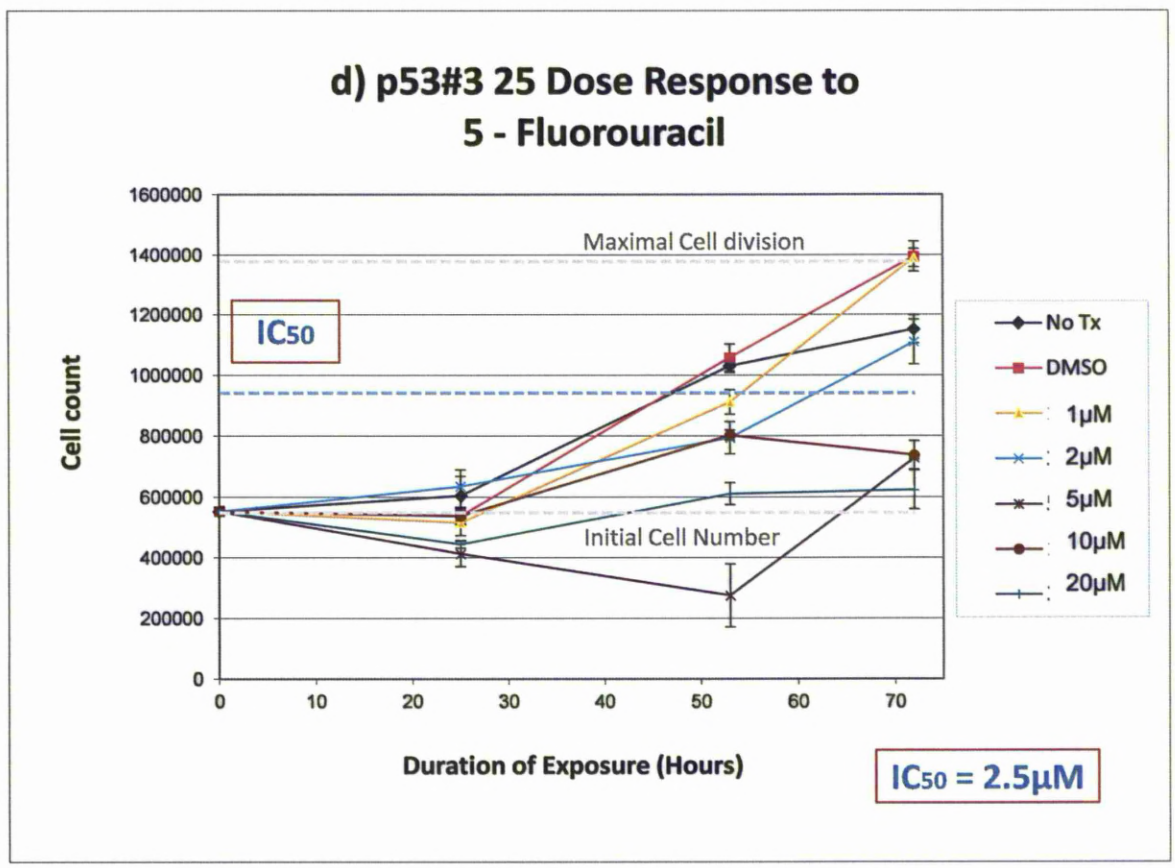
## 7.3 Determination of the 5FU dose response in p53 suppressed pSUPER cell lines

Having established the $IC_{50}$ for these two chemotherapeutic agents, this value was then used to guide the range of doses appropriate to study in a selection of the pSUPER clones. Initially 5 fluorouracil was applied at [1 – 20μM] to a sample of clones to see if this altered proliferation rates and hence $IC_{50}$. pSUPER clones were selected for study which had been transfected with the p53#3 mutant specific shRNA and demonstrated varying degrees of mutant p53 knock down when visualised by western blot. Hence a cell line with no visible knock down (p53#3 22), a 50 - 75% reduction from baseline (p53#3 25) and two cell lines with >75% reduction in mutant p53 levels (p53#3 19 and p53#3 33) were selected, so that the specificity of any alteration in $IC_{50}$ could be correlated with p53 status (as previously studied in section 5.6). All the selected clonal lines also showed GFP expression, to help confirm that p53 suppression was likely due to the vector insert rather further potentially oncogenic, genetic or epigenetic changes resulting from clonal selection.


a) SW620 Dose Response to
5 - Fluorouracil
Maximal Cell division
IC50
Initial Cell Number
Cell Count
Duration of Exposure (Hours)
No Tx
DMSO
1μM
2μM
5μM
10μM
20μM
IC50 = 2 - 5μM


b) p53#3 19 Dose Response to
5 - Fluorouracil
Maximal Cell division
IC50
Initial Cell Number
Cell Count
Duration of Exposure (Hours)
No Tx
DMSO
1μM
2μM
5μM
10μM
20μM
IC50 = 5 μM


c) p53#3 22 Dose Response to 5 - Fluorouracil
Cell Count
3000000
2500000
2000000
1500000
1000000
500000
0
Maximal Cell division
IC50
Initial Cell Number
0
10
20
30
40
50
60
70
Duration of Exposure (Hours)
No Tx
DMSO
1µM
2µM
5µM
10µM
20µM
IC50 = 5 µM


d) p53#3 25 Dose Response to 5 - Fluorouracil
Cell count
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
Maximal Cell division
IC50
Initial Cell Number
0
10
20
30
40
50
60
70
Duration of Exposure (Hours)
No Tx
DMSO
1µM
2µM
5µM
10µM
20µM
IC50 = 2.5µM

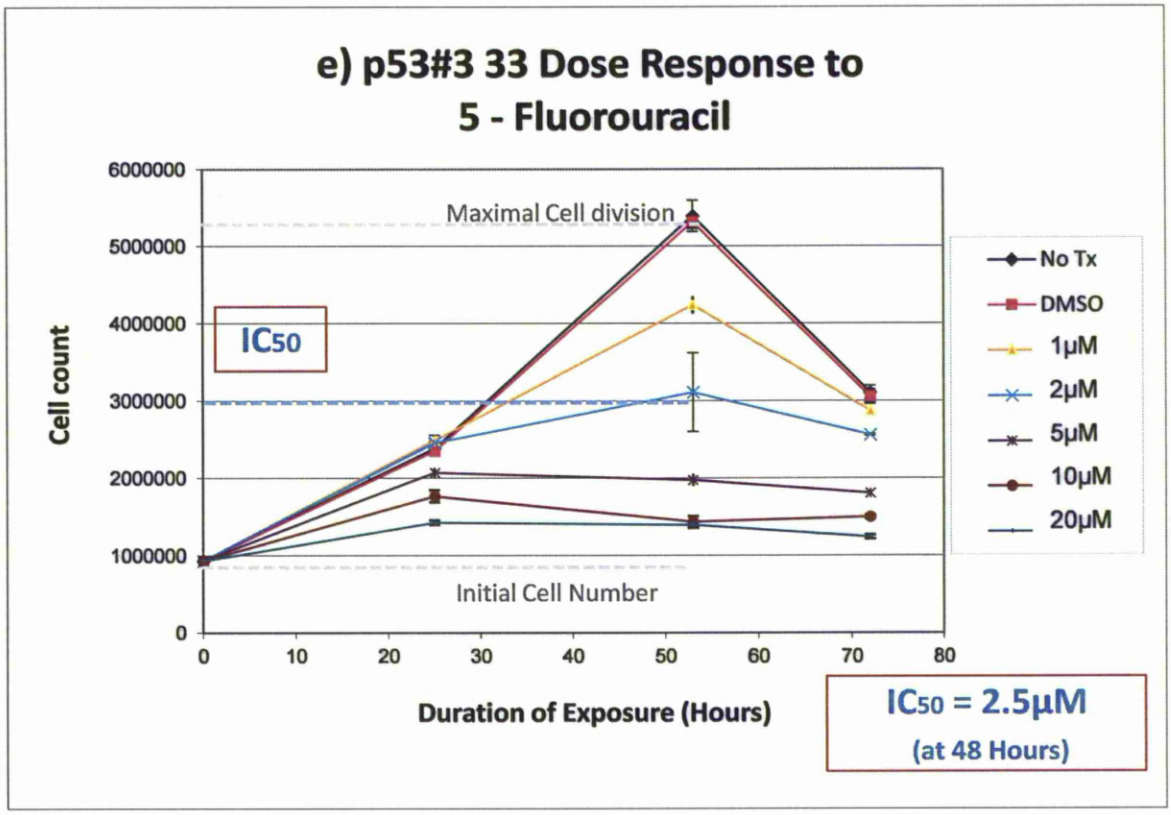



***Figure 7.3 (a-e): The 5FU dose dependant response in p53 suppressed pSUPER cell lines***

The first observation from Figure 7.3a is that the dose - response relationship in the parental SW620 cell line differs from that previously discussed in Figure 7.1. The $IC_{50}$ dose of 2μM shows an increased amount of cell proliferation, when compared with the DMSO control, in this instance. This seems inconsistent with the rest of the experiment, which demonstrates the expected dose dependent division restriction and for this reason the $IC_{50}$ could not be more accurately calculated then the range 2 - 5 μM. It is clear that this experiment requires repeating, with the most likely explanation for the anomalous result being a technical error or chemo resistance.

The p53#3 19 and 22 cell lines, figures 7.3b and 7.3c demonstrate a reduction in proliferation in a dose related manner as expected, with $IC_{50}$ value of 5μM for both

these clonal populations. Since the p53#3 19 cell line has >75% suppression of mutant p53 expression, whereas the p53#3 22 cell line has no clearly detectable reduction in p53 level, this seems to suggest that mutant p53 suppression is not correlated with a reduction in cell proliferation in these particular clones.

The p53#3 25 cell line, figure 7.3d, shows a reduction in cell number with the 5μM dose at 48 hours, which is greater than seen with the higher doses, but this rectifies at 72 hours producing an IC50 of 2.5μM and this is clearly a consequence of technical variability.

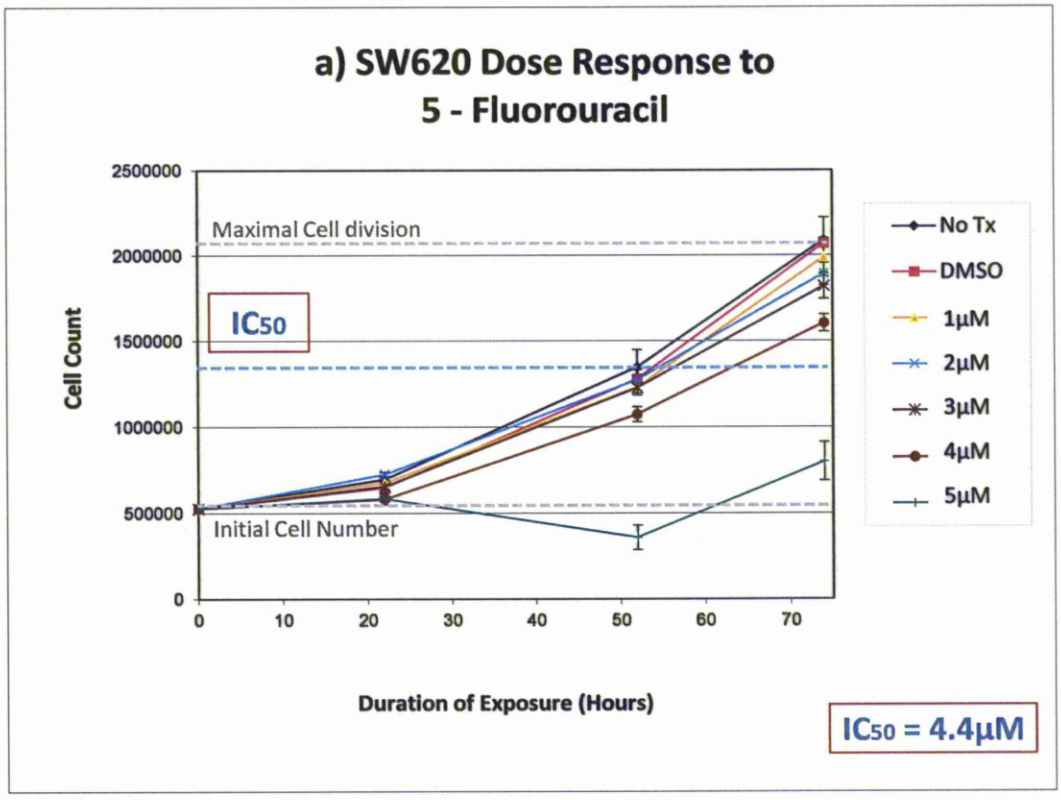
In the p53#3 33 cell line, figure 7.3e demonstrates a significant growth restriction across all experimental conditions at 72 hours. This correlated with a visible change in cell morphology, with cells appearing rounded and less cohesive than previously and hence the results at this time period were discarded, with the 48 hour figure used to calculate the $IC_{50}$ value of 2.5μM.

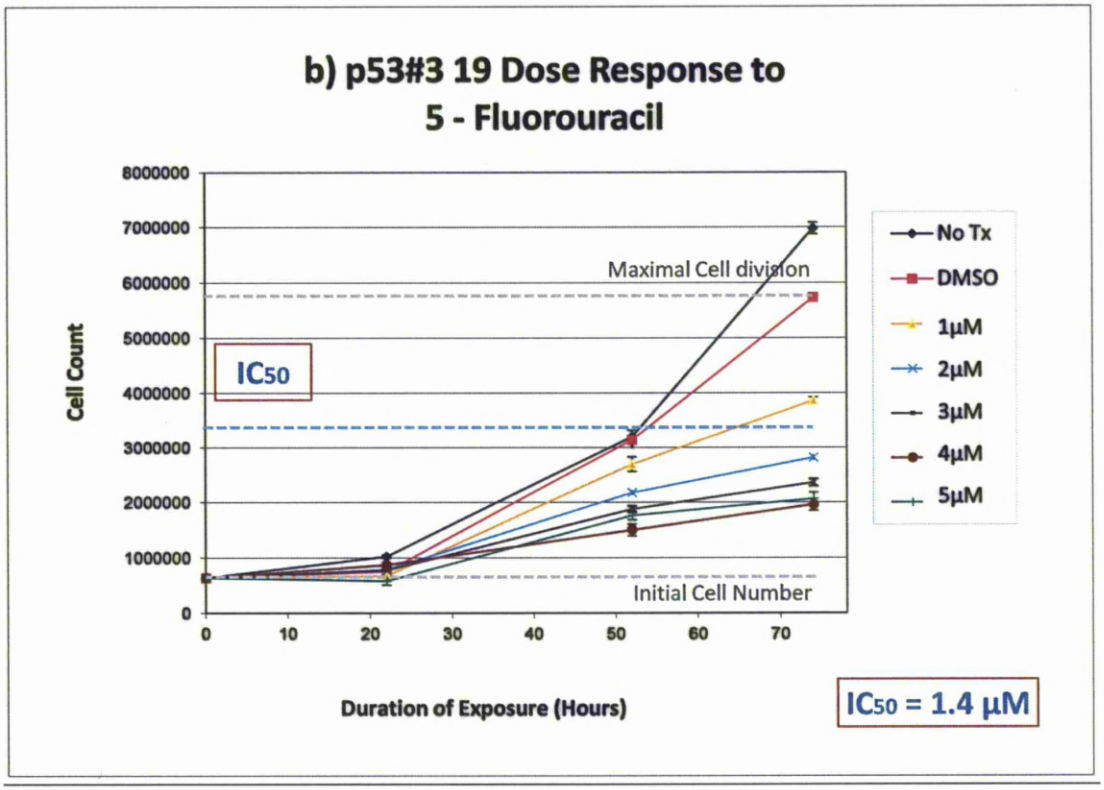
The $IC_{50}$ values of 2.5μM for both the mutant p53 suppressed clones shown in Figures 7.3d and 7.3e (p53#3 25, 50-75% reduction, p53#3 33, >75% reduction), does suggest correlation with the hypothesised findings of reduced cell proliferation with mutant p53 suppression in these particular clones, but as outlined above this finding was not consistent across our panel of clones reviewed.

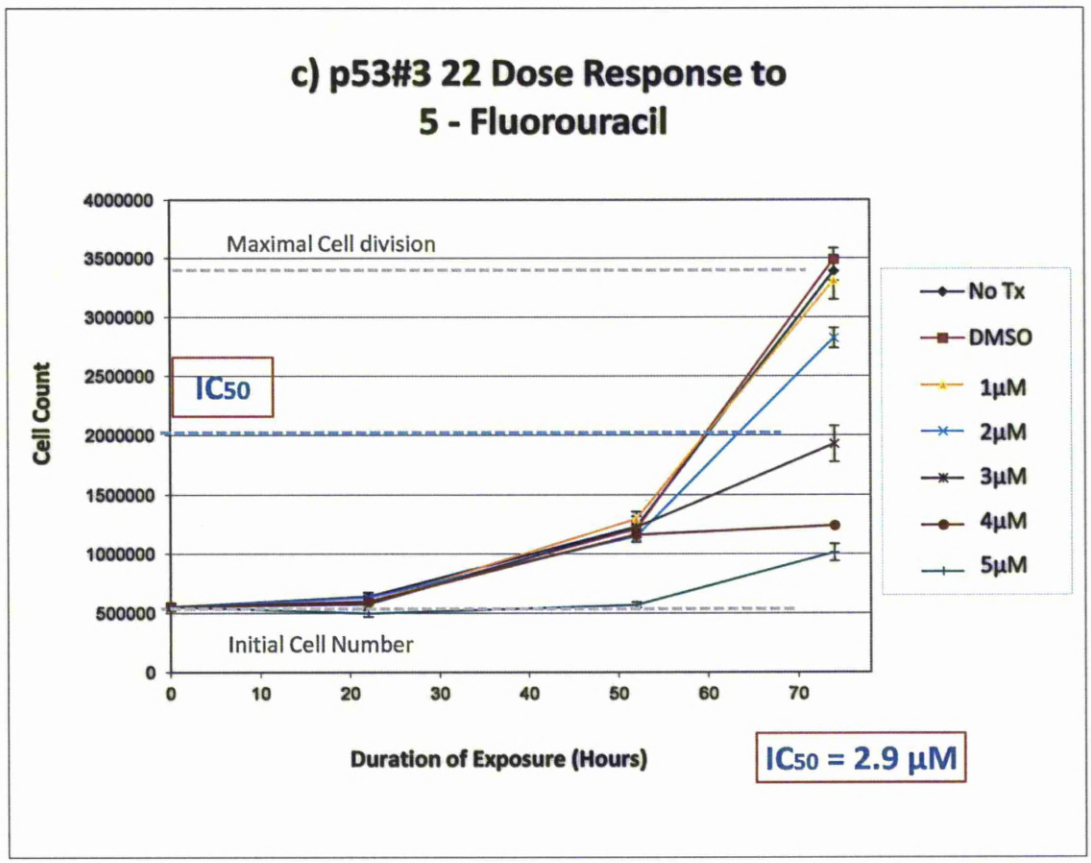
As a result of the seemingly outlying 2μM result in the parent cell line, combined with the discarded 72 hour results in the p53#3 33 cell line, this entire experiment was repeated using the same choice of p53 clones. This would also allow

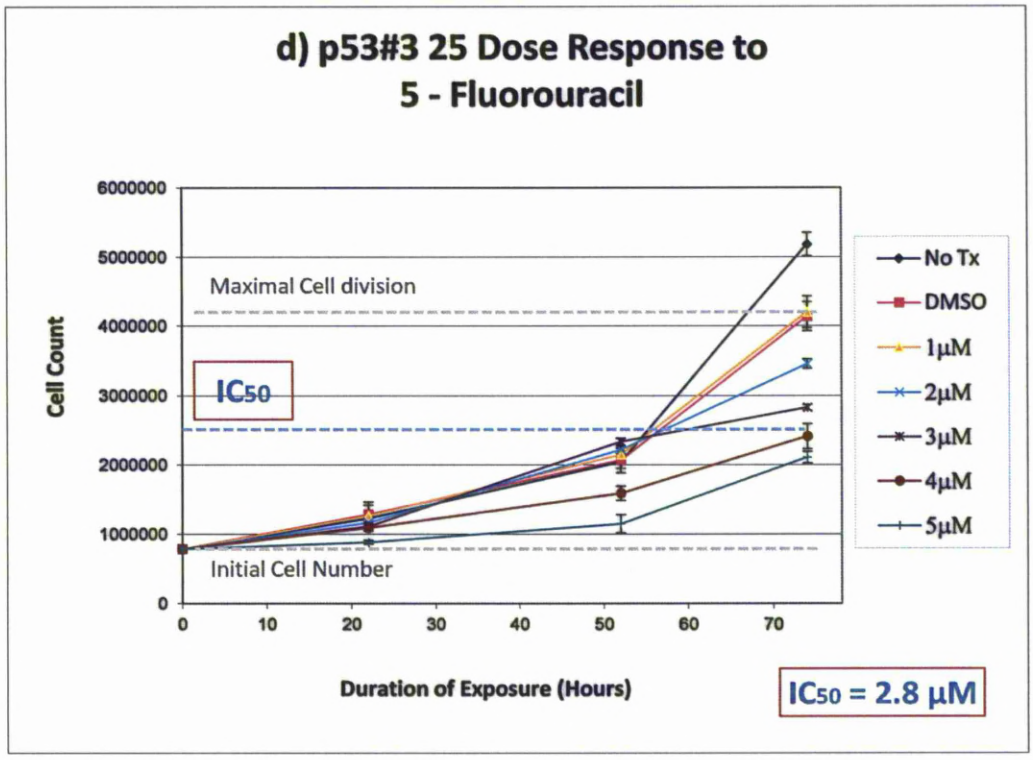
collaboration, or otherwise, of the lack of correlation between mutant p53 suppression and the resultant $IC_{50}$ dose.

In the repeat experiment the range of 5FU applied to the cells was narrowed, since previous values had all been $IC_{50} \leq 5$, it was felt the results would be more accurate if further values in the range 0 – 5μM were studied, whereas larger doses would add little more usable data. The results of this repeated experiment displayed in Figure 7.4:




b) p53#3 19 Dose Response to
5 - Fluorouracil
Cell Count
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
Maximal Cell division
IC50
Initial Cell Number
0
10
20
30
40
50
60
70
Duration of Exposure (Hours)
No Tx
DMSO
1µM
2µM
3µM
4µM
5µM
IC50 = 1.4 µM


c) p53#3 22 Dose Response to
5 - Fluorouracil
Cell Count
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Maximal Cell division
IC50
Initial Cell Number
0
10
20
30
40
50
60
70
Duration of Exposure (Hours)
No Tx
DMSO
1µM
2µM
3µM
4µM
5µM
IC50 = 2.9 µM



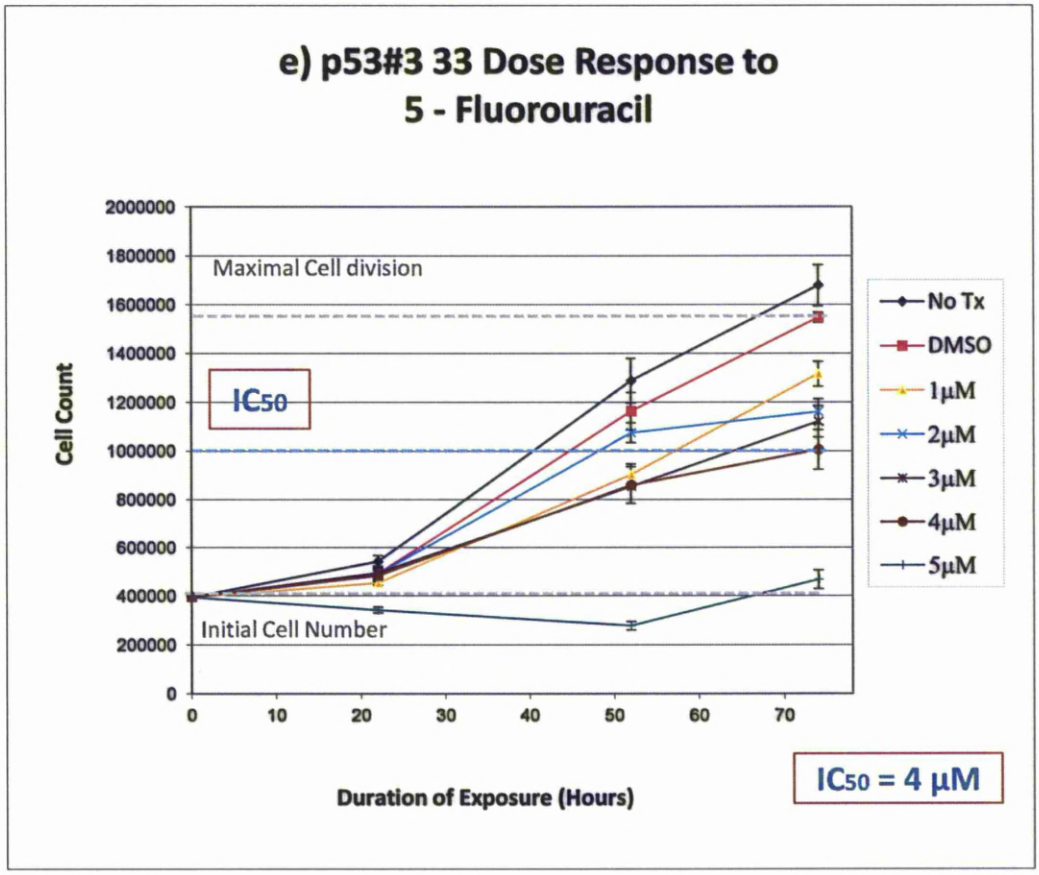




*Figure 7.4 (a-e): Experiment 2: Comparison of the 5FU Response in a Selection of Mutant p53 Suppressed pSUPER Cell Line*

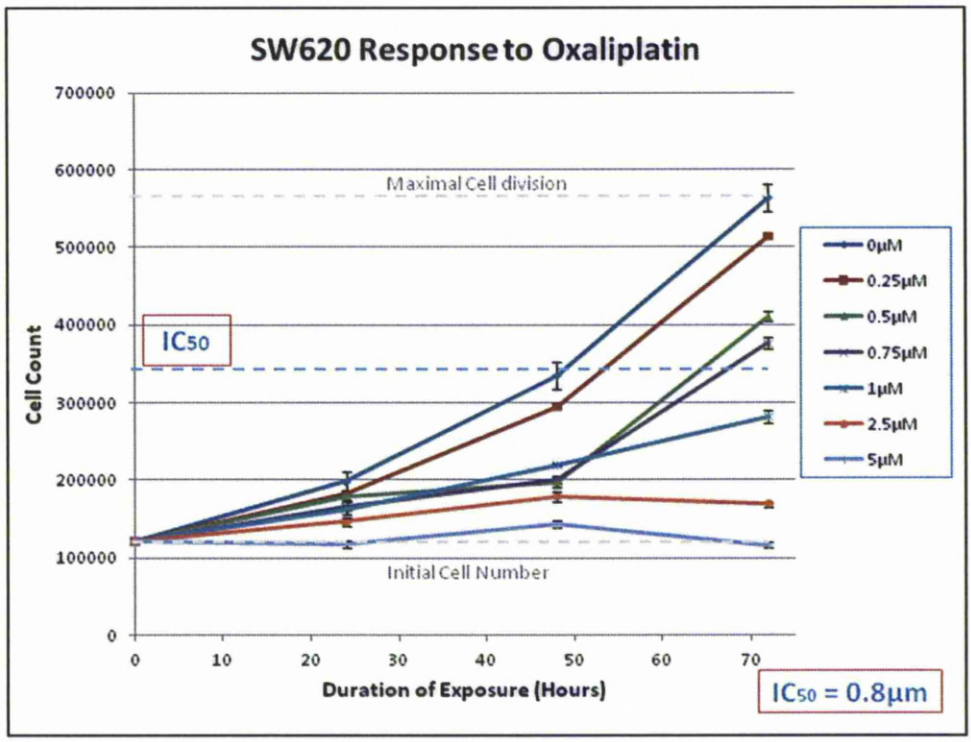
## 7.4 Experiment 2: The 5FU Response in a Selection of Mutant p53 suppressed pSUPER cell lines

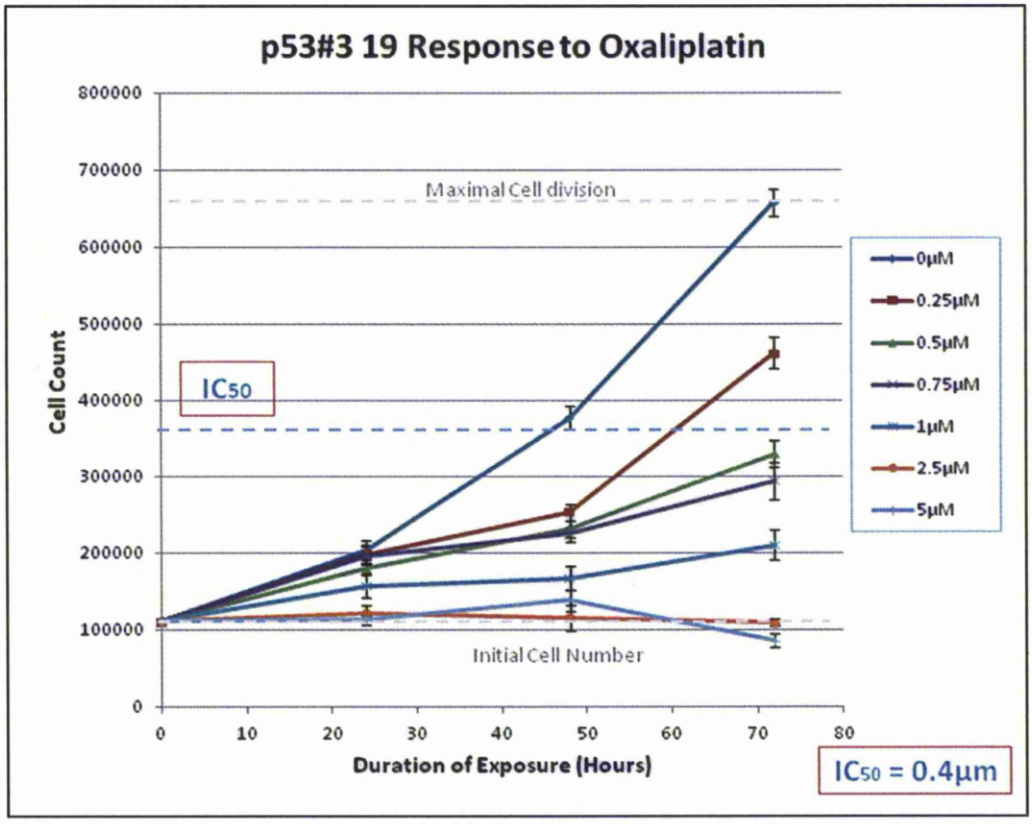
In Figure 7.4a the dose-response relationship in the SW620 parent cell line follows the expected pattern of proliferation restriction with increasing drug concentrations, and an approximate $IC_{50}$ of 4.4μM is observed. This differs from the value of 2μM ascertained in Figure 7.1. This relatively small difference may be explained by the differing starting cell numbers at time zero, with a two-fold difference between the two experiments. Logically initial cell confluence might be expected to have an impact on $IC_{50}$, therefore it was important to include a parental cell line control for each experiment and to try as far as possible to keep starting cell numbers uniform between serial experiments to allow valid comparisons.

The p53 suppressed pSUPER mutants all show a reduction in $IC_{50}$ when compared to the parent cell line in this experiment with values of 1.4μM (p53#3 19,figure 7.4b), 2.8μM (p53#3 25,figure 7.4d) and 4μM (p53#3 33, figure 7.4e). However the reduction was not proportional to the degree of suppression visualised by western blot and the cell line with no visible knock down (p53#3 22) also had a lower $IC_{50}$ than the parental cell line at 2.9μM, figure 7.4c.

## 7.5 The Oxaliplatin Dose Dependent Response in P53 Suppressed pSUPER Cell lines

The same experimental design was utilised with a second chemotherapeutic agent, Oxaliplatin, which was applied in the range 0 – 5 μM. Due to time constraints only two clones were studied, both with > 75% reduction in p53 expression, with the results demonstrated in figure 7.5:


SW620 Response to Oxaliplatin
Maximal Cell division
IC50
Initial Cell Number
Cell Count
Duration of Exposure (Hours)
0μM
0.25μM
0.5μM
0.75μM
1μM
2.5μM
5μM
IC50 = 0.8μm


p53#3 19 Response to Oxaliplatin
Maximal Cell division
IC50
Initial Cell Number
Cell Count
Duration of Exposure (Hours)
0μM
0.25μM
0.5μM
0.75μM
1μM
2.5μM
5μM
IC50 = 0.4μm

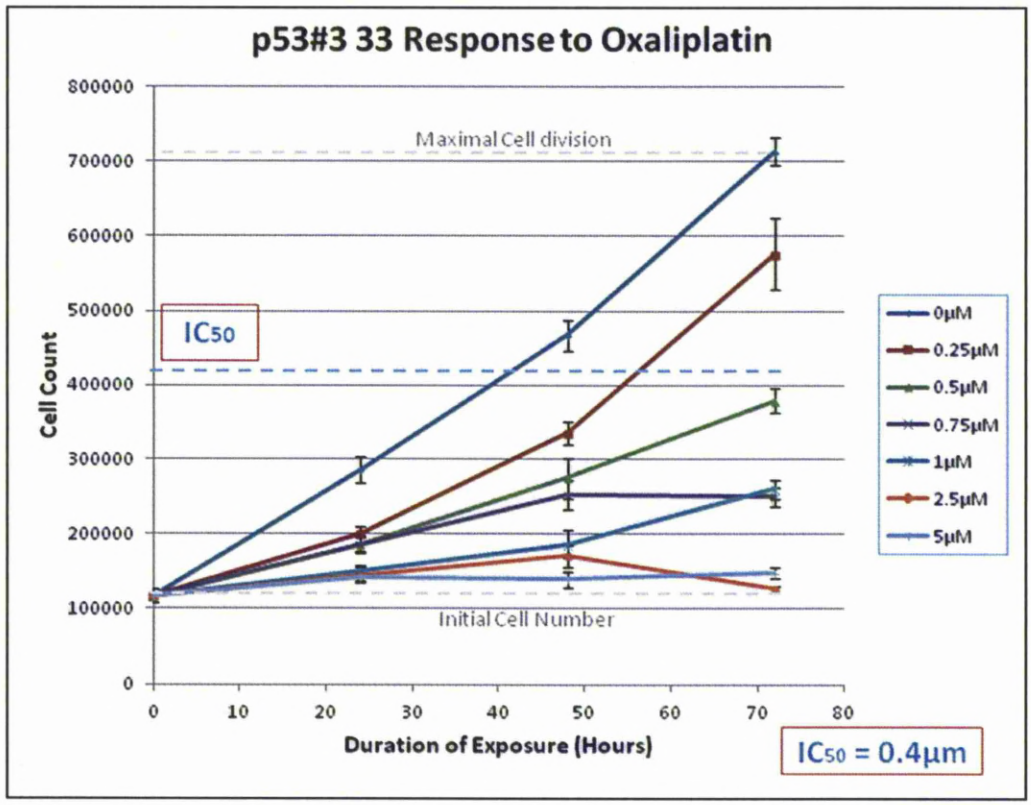



***Figure 7.5: The Oxaliplatin Dose - Response in p53 Suppressed pSUPER Cell Lines***

Figure 7.5 demonstrates an $IC_{50}$ of 0.8μM in the SW620 parent cell line, which is comparable to the initial value 0f 0.9μM obtained previously in Figure 7.1. This suggests that the experimental system has some validity and that results obtained from these experiments are reasonably reliable.

This $IC_{50}$ value is halved in both the mutant p53 suppressed cell lines studied, with both graphs showing good dose-response and there are no major outliers or other obvious anomalies.

## 7.6 Summary of the $IC_{50}$ values for 5 fluorouracil and oxaliplatin

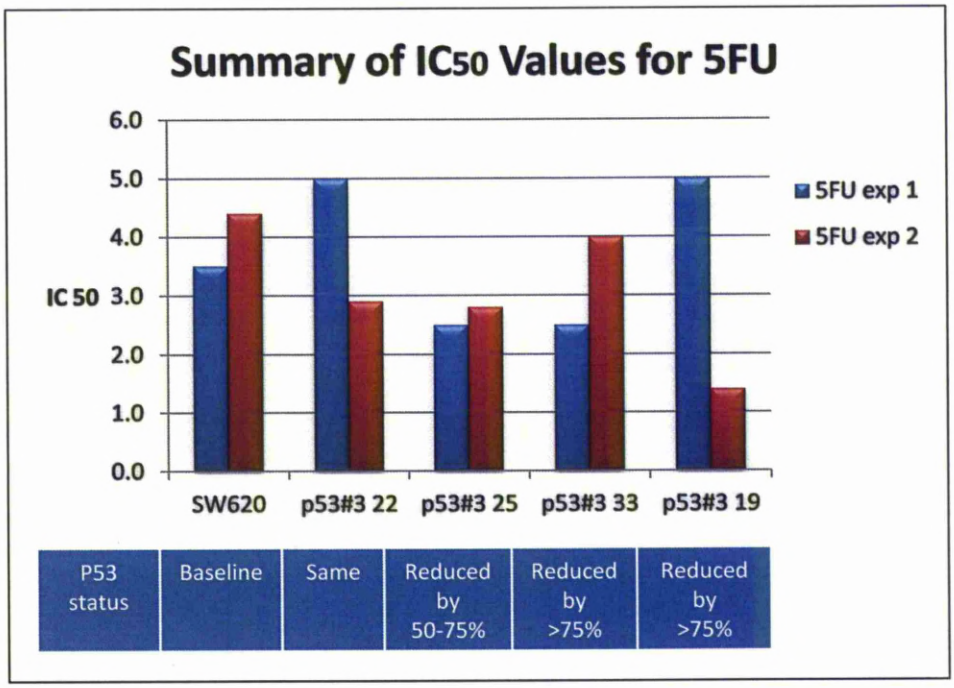



**Figure 7.6: Summary of drug sensitivity data for 5 -fluorouracil**

Figure 7.6 summarises the results of Figures 7.3 and 7.4 alongside the p53 status of each cell line and demonstrates that whilst there is a general trend towards $IC_{50}$ reduction with p53 suppression, there are anomalies and variation between the experiments as outlined earlier, in section 7.3.

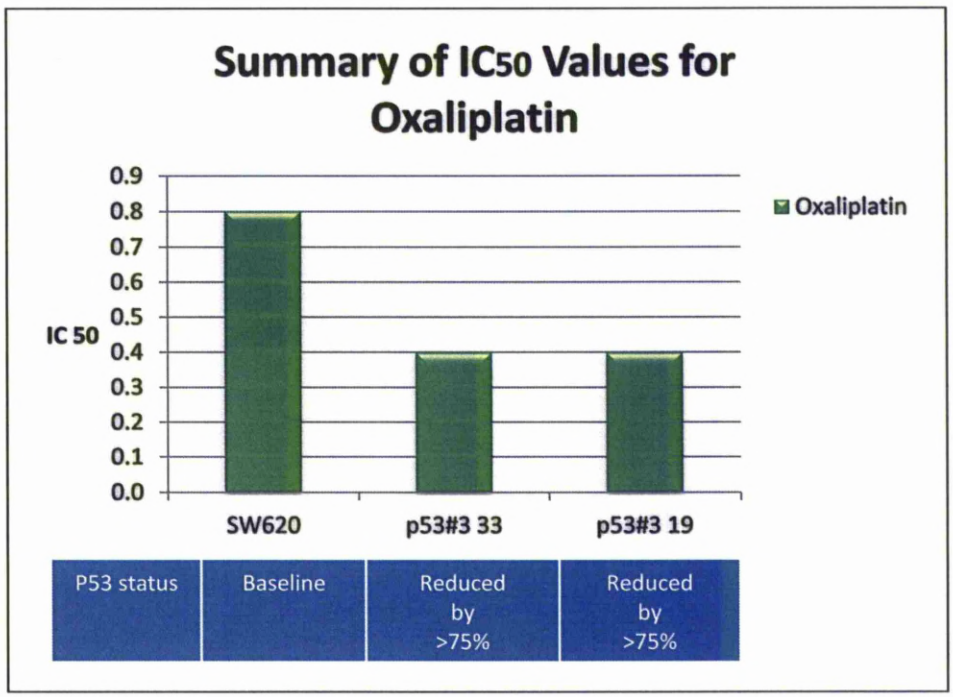



***Figure 7.7: Summary of drug sensitivity data for Oxaliplatin***

Figure 7.7 demonstrates the lower IC50 levels for oxaliplatin with both the mutant p53 suppressed SW620 populations studied. From these data it appears that the use of a lower Oxaliplatin concentration results in a comparable reduction in cell proliferation in the mutant p53 shRNA cell lines tested and thus it appears that mutant p53 may contribute to resistance to oxaliplatin in these cells. However, the previous experiment has demonstrated that caution is required when extrapolating beyond results in any given cell line and many clones must be tested with more replicates to confirm this finding.

# Chapter 8. Conclusions and Discussion

It was established in the introduction that colorectal cancer remains a common disease and even with advances in surgical techniques and adjuvant therapies, five year survival is still only in the region of 50%. Possible target areas for improving this mortality figure include: primary prevention strategies, screening programmes to increase early stage disease diagnosis and improved adjuvant therapies. Currently chemotherapy regimens are proven to confer an additional survival advantage of as much as 13% in node positive disease when compared with surgery alone [80]. Whilst chemotherapy as a palliation in advanced disease can provide three to six months extra life expectancy with concurrent maintenance of quality of life [87]. However, the current chemotherapeutic agents are recognised as having a relatively non-specific anti-tumour mechanism, simply targeting the rapidly dividing cancer cells preferentially, but also damaging healthy tissue and hence have considerable toxicity and side effect profiles. As knowledge of the role of genetics in carcinogenesis has become increasingly understood and delineated, so has the search for molecularly targeted 'gene therapy' drugs. In colorectal cancer the monoclonal antibody Bevacizumab (Avastin) has already been utilised clinically to confer a 4.7 month increase in median survival compared with combination chemotherapy in the first line treatment of KRAS wild-type tumours [89]. Many believe the future of adjuvant cancer treatment lies in such targeted therapies, whereby an individual would have their tumour analysed for its genetic mutations and an individual therapy based on targeting some of these specific mutations would be administered. Colorectal cancer provides an attractive disease for such targeted therapies, since much is understood about the multiple genetic pathways that are implicated in the

disease's progression. The step – wise accumulation of genetic defects from early adenoma to late invasive and metastatic cancer provides differing known target genes at varying stages of the malignant process. Since p53 mutations have a vital role to play in the late stages of this transformation from adenoma to carcinoma, this obviously provides an important potential clinical target since it is at this advanced state of carcinogenesis that adjuvant therapies currently have their clinical role. p53 mutations are also a relevant potential clinical target due to their frequency of occurrence, i.e. their presence in almost half of all colorectal cancers [172]. p53 mutations have been linked to altered response to current chemotherapeutic agents. It has already been established that the effects of chemotherapy on survival are influenced by p53 status and that chemotherapeutic resistance is associated with expression of mutant p53 proteins [194, 195].

On this basis, the hypothesis, that inhibiting mutant p53 in colorectal cancers may reduce malignant potential and augment response to adjuvant therapies, was established.

The first step experimentally towards answering this question was to grow in tissue culture a panel of transformed colorectal cancer cell lines, expressing both mutant and wild-type p53, on which to test this hypothesis. Baseline p53 levels for the cell line cohort were studied and compared with the published data. It was demonstrated that p53 protein levels were many magnitudes higher in the mutant cell lines than in those documented to express the wild-type protein. A previously published study suggested that this accumulation is likely due to the prolonged half-life of the mutant protein, since it is outside the usual MDM2 mediated negative feedback loop [204].

This became relevant when trying to suppress and study the effects of mutant p53 utilising transient siRNA technology. We established that whilst wild-type p53 could be visibly significantly reduced, the mutant protein proved harder to down-regulate, probably as a result of the far greater amount of the protein product present.

In order to try and improve mutant p53 suppression using siRNA sequences, a series of optimisation experiments were undertaken. Despite modification of several variables within the transfection process, levels of down regulation of the mutant p53 protein remained sub-optimal. This could be explained to some degree by the fact that even if 100% of cells underwent successful transfection with the siRNA sequence, the degree of suppression would still depend on the efficacy of the actual siRNA mediated down regulation of the target protein within any given cell. This, coupled with the high expression levels of the mutant protein, lead to the conclusion that the manufacture of stable shRNA expressing clones would be necessary to effectively and consistently study the effects of any down regulation of the mutant p53 protein.

Another consideration in our initial siRNA experiments was to ascertain whether any specificity could be obtained for targeting a mutant p53 sequence whilst protecting wild-type p53 protein production. Clinically, this is important, since ideally any therapy should target only the mutant protein avoiding any off target effects on functioning wild-type p53 within neighbouring healthy cells. In 2002, Martinez claimed that a single base difference in siRNAs could discriminate between mutant and wild-type p53 in cells expressing both forms, resulting in the restoration of wild-type protein function [244]. In order to confirm the specificity of siRNA targeting,

three R273H mutant specific siRNA sequences were designed and tested alongside one wild-type specific sequence in cell lines expressing either mutant or wild-type p53 protein. This mutation was chosen since it is one of the commonest seen in colorectal cancer (~6% of cases) and was present in all of our p53 mutant colon cancer cell lines [183]. One of the three siRNA mutant sequences designed did show partial protection of the wild-type protein product, but this effect was not witnessed with the other two sequences. Therefore it may be possible to design an siRNA oligonucleotide that is selective for a specific p53 mutant and protective of the wild-type. However, our findings were not as conclusive as those presented by Martinez and suggested that siRNA sequence design may be crucial in conferring such specificity. Subsequently shRNA inserts were designed for insertion into the pSUPER vector from the mutant p53 siRNA sequence that had shown a degree of specificity.

Brummelkamp *et al.* [254] initially published in 2002 that a new vector system (pSUPER, oligoengine) could cause efficient and specific down regulation of genes by directing intra-cellular siRNA synthesis. The incorporation of antibiotic resistance into the plasmid allows selection of only those clones in which the plasmid had become successfully integrated, thus overcoming the previously discussed issues of transfection efficiency. Transfection followed by antibiotic selection allowed clonal populations of cells to be produced which had the pSUPER plasmid containing specific shRNA expressing sequences incorporated into their genome. It became apparent early in the analysis of the selected clonal populations, that not all of the clones grown under antibiotic selection displayed the desired down regulation of the

target mutant p53 protein. Hence, large numbers of clonal populations had to be screened to compensate for this inter clonal variability.

In total over one hundred clones were produced and analysed, containing empty vector and scrambled sequence controls as well as those with the wild-type and R273H mutant specific shRNA incorporated. Clonal populations were created in three separate cell lines, with successful down regulation of mutant p53 protein seen in all. The levels of suppression achieved were far greater than seen in our earlier transient studies and compared favourably with the 20-50% down regulation reported in similar work on mutant p53 in colon cancer published by Bossi *et al* in 2006 [249].

Subsequently further experiments were conducted to assess the impact of this down regulation on cellular function. The effect of mutant p53 suppression on the rate of cell proliferation in the SW620 cell line was studied by comparing the increase in cell number over time of various pSUPER clones. Whilst some of the clonal populations with down regulation of mutant p53 expression did show a reduced proliferation rate, this was not consistent across all suppressed clones and some of the clones with no alteration in mutant p53 levels also exhibited slowed proliferation. This contradicts the findings of Bossi *et al.* who demonstrated reduction in both cell numbers and cell viability in all three of the mutant p53 colon lines they studied. However, we have previously discussed the degree of inter-clone variability witnessed in our studies and this may account for the discrepancies between our findings and those published by Bossi *et al.*

Further analysis of cellular function was not possible within the time constraints of this study, however there is considerable scope for further assays on the down regulated mutant p53 clones. Alterations in cell cycle progression could be studied with Brd U incorporation to assess the percent of cells in S phase. FACS (Fluorescence Activated Cell Sorting analysis) could be utilised to study whether cellular apoptosis rates are affected by down regulation of the mutant p53 protein. The effect of down regulating the mutant protein on some of the so called 'gain of function' target genes could also be studied.

However, the primary aim of this study was to ascertain if any clinical benefit could be gained by reducing mutant p53 in colorectal tumours. The next set of experiments undertaken considered the response of the mutant p53 suppressed clones to two chemotherapeutic agents used currently as adjuvant and palliative treatments in the management of colorectal cancer (5 fluorouracil and oxaliplatin).

Two SW620 clones with significant suppression of their mutant p53 protein levels demonstrated $IC_{50}$ levels half that of the parent population when treated with oxaliplatin. Thus, half the amount of chemotherapeutic agent was needed to produce the same effect when the mutant p53 protein had been suppressed. The results with the second agent, 5 fluorouracil, were less consistent. Whilst there was a general trend towards lower $IC_{50}$ values in the down regulated clones, this was not always the case. The overall impression from our work is that suppression of mutant p53 may well improve the response of colorectal cancer cells to chemotherapy, especially oxaliplatin. However, these experiments show that extrapolating beyond results in

any given clone can be misleading and that large numbers of clone replicates must be studied before any appropriate conclusions can be drawn.

One mechanism for confirming the specificity of any mutant p53 mediated effects would be to re-establish mutant p53 expression in the suppressed clones showing altered phenotypes and considering if the observed effect is consequently reversed. In order to do this, a plasmid was designed that expresses the R273H sequence, but has base pair mutagenesis in the region targeted by our shRNA expressing vector, so that the amino acids are coded to produce the same protein product, but not targeted by the shRNA. Completion of this Polymerase Chain Reaction mediated site mutagenesis was not possible within the time constraints of the laboratory work, but its potential to confirm causality would add greater credence to the specificity of the described mutant p53 mediated effects. This preliminary work is detailed in Appendix A.

In summary, this thesis demonstrates the value of utilising the pSUPER vector to provide efficient and stable protein suppression, even in the context of very high levels of over-expression. However it also highlights that caution should be taken when drawing conclusions due to the variability present between clonal populations. This could potentially be due to further genetic mutations occurring within the clonal population during the selection and culturing process.

Whilst the technology of RNA interference provides a useful tool to study gene silencing in the context of transformed colorectal cancer cells in culture, it is important to consider whether this could ever translate into a clinical therapy. The

main issue with its use as an adjuvant therapy in colorectal cancer is the mode of delivery to tumour tissue. The purpose of adjuvant therapy is to 'mop up' any residual tumour cells that may reside within the draining lymph nodes, the peritoneal cavity or as micro-metastases, primarily within the liver. As such, any therapy should ideally be delivered systemically, to allow tumour cells at multiple sites to be targeted. Unfortunately SiRNAs are unstable in the serum environment where they can be degraded by RNAase and are rapidly excreted in the urine if delivered systemically. Overcoming multiple tissue barriers and ensuring efficient endocytosis into the target cell are also essential to successful delivery. The use of cationic lipids and polymers as siRNA delivery carriers have been shown to help overcome some of these obstacles but as of yet no successful systemic therapies utilising RNA interference are available.

There are other methods of effectively silencing gene products which are systemically deliverable and utilised in clinical practise at present. Monoclonal antibodies have already been utilised alongside adjuvant chemotherapy regimes in colorectal cancer in the form of the anti VEGF agent Bevacizumab (Avastin). If further studies were to confirm the benefits of down regulation of mutant p53, then manufacturing a monoclonal antibody against such proteins would seem a reasonable clinical target. One challenge would be the variability of mutations present, necessitating multiple compounds for different patients. However the presence of common hot-spot mutations reduces the number of alternatives required to some degree. Another major concern would be agent specificity; with protection of normal p53 function in healthy tissue whilst silencing the mutant protein within tumour cells. This is a difficult technical problem since most mutations have only a single base

pair substitution from the wild-type sequence. A possible way to overcome this would be to look further into the mechanism of mutant p53 gain of function and in particular consider whether any of the target genes further down the pathway may be potential therapeutic targets.

# Appendix A:

# Polymerase Chain Reaction mediated site mutagenesis

**Introduction**

One mechanism for confirming the specificity of any mutant p53 mediated effects would be to re-establish mutant p53 expression in the suppressed clones showing altered phenotypes and considering if the witnessed effect is reversed. A plasmid was designed that expresses the R273H mutant p53 sequence, but has base pair mutagenesis in the region targeted by our shRNA expressing vector, so that the amino acids are coded to produce the same protein product, but not targeted by the shRNA mediated suppression.

**Hypothesis**

Any alterations in cell proliferation, or response to chemotherapy, in cells transformed with the pSUPER plasmid expressing R273H shRNA, may be the result of the cloning process itself, rather than the specific desired shRNA effect i.e mutant p53 suppression. Reintroduction of the mutant R273H protein into these cells would permit testing of the specificity of any suppression effects, by demonstrating whether phenotypic reversal occurred when the same protein is transcribed from the altered DNA sequence.

**Primer Design**

To perform PCR mutagenesis four primers were designed using three sequential PCR reactions as outlined in Figures 0.1 to 0.6:

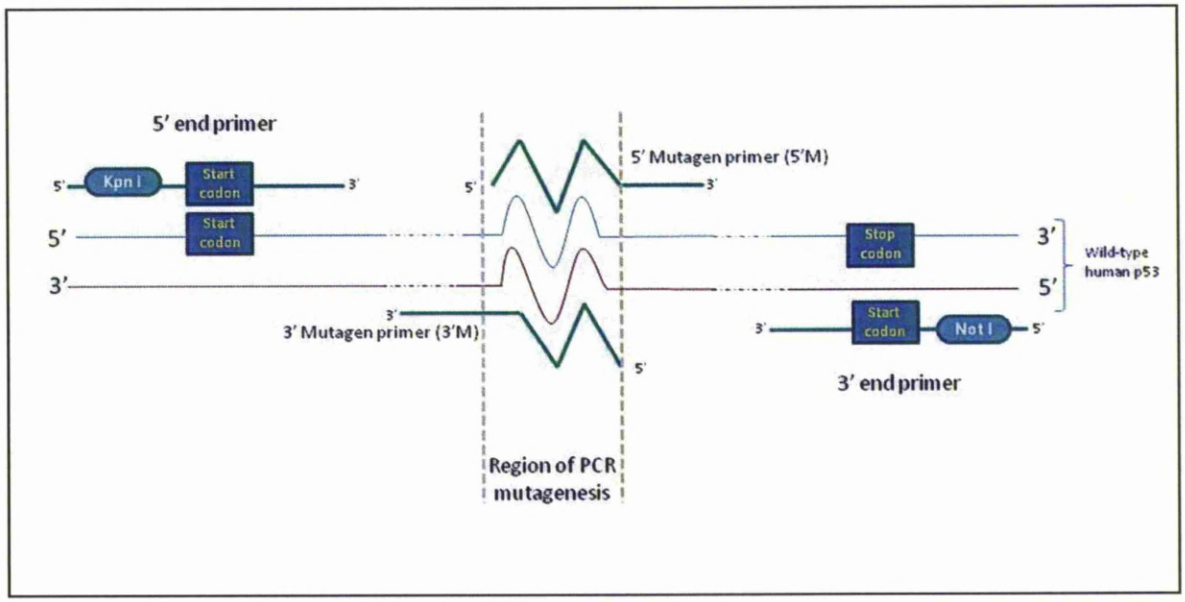



*Figure 0.1: Target sites for the four primers*

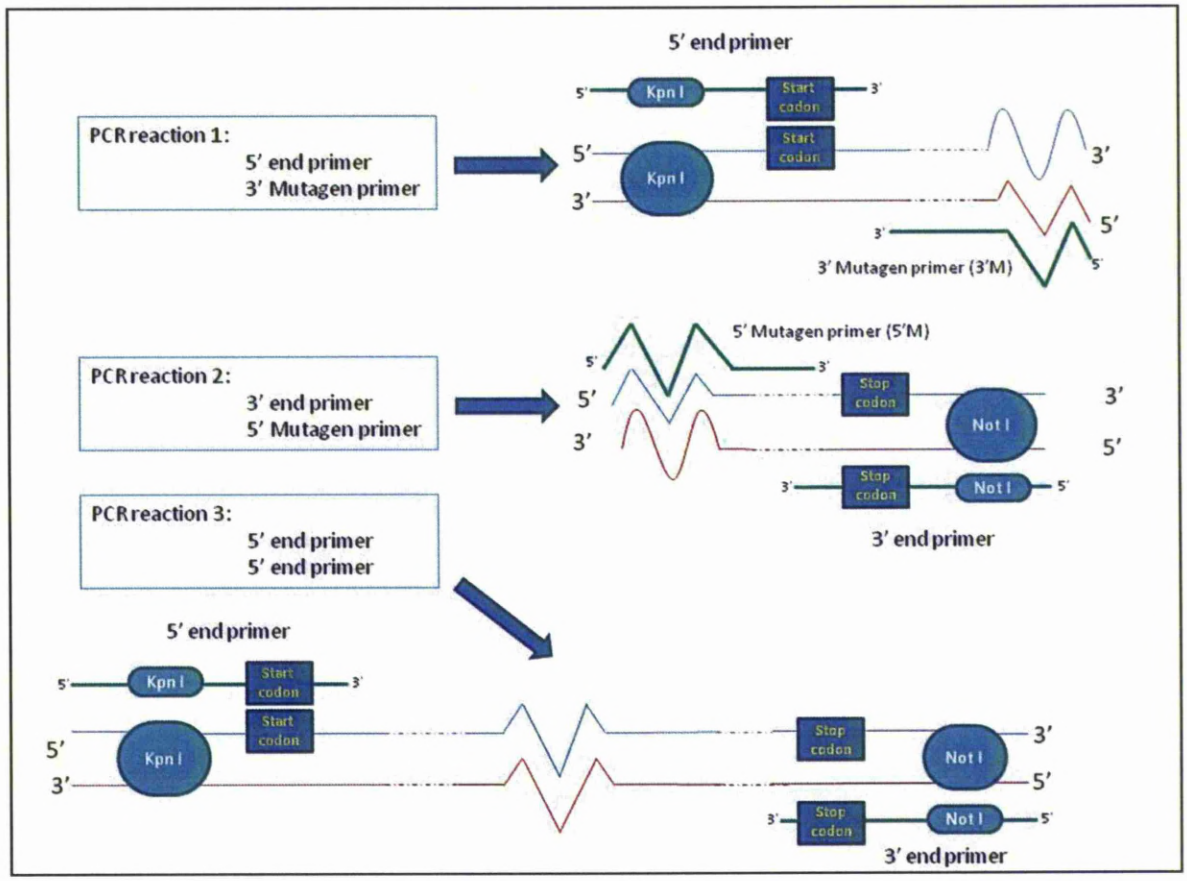



*Figure 0.2: Strategy for PCR mutagenesis*

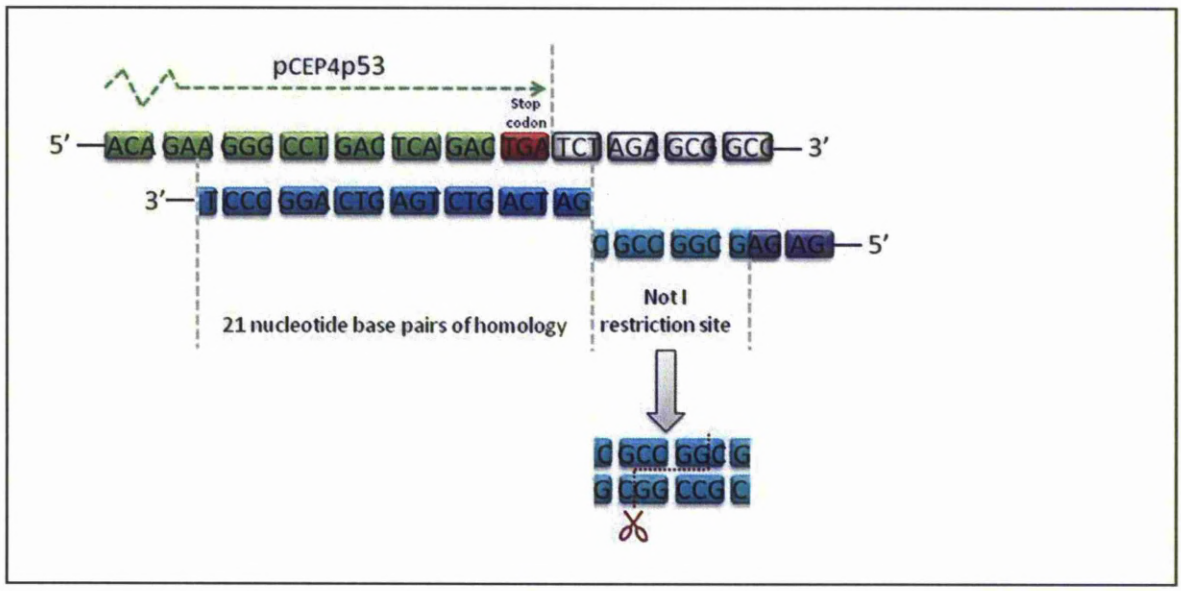



*Figure 0.3: Design of the 3' end primer for pCEP4p53*

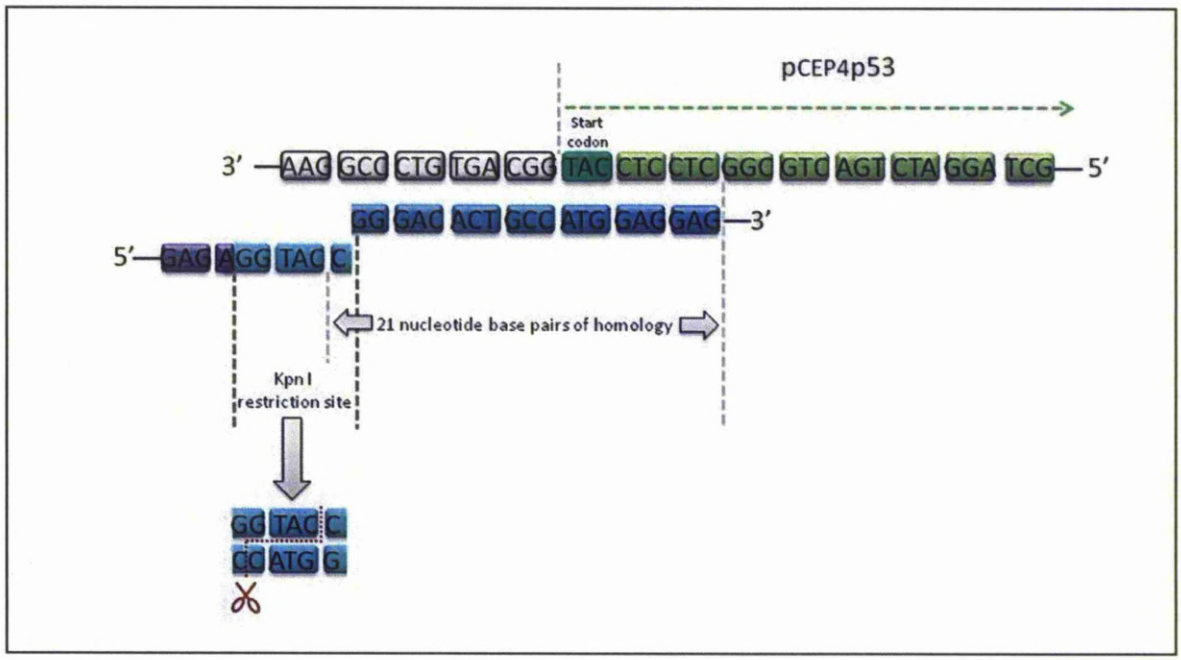



*Figure 0.4: Design of the 5' end primer for pCEP4p53*

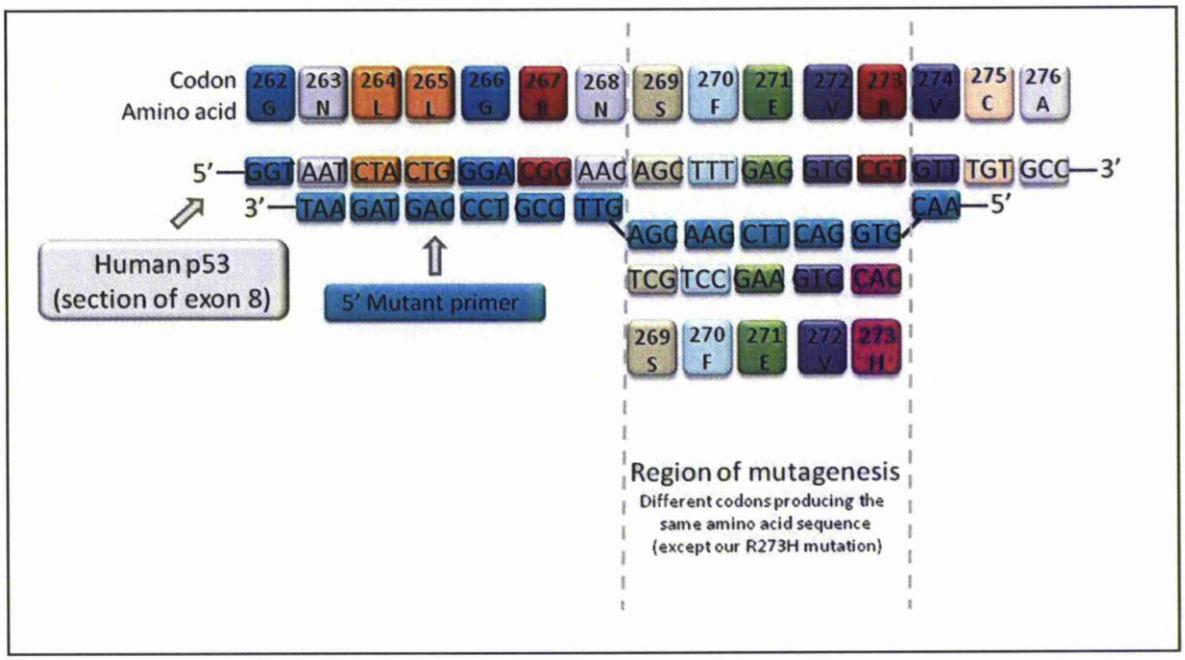



*Figure 0.5: Design of the 5' Mutant for pCEP4p53*

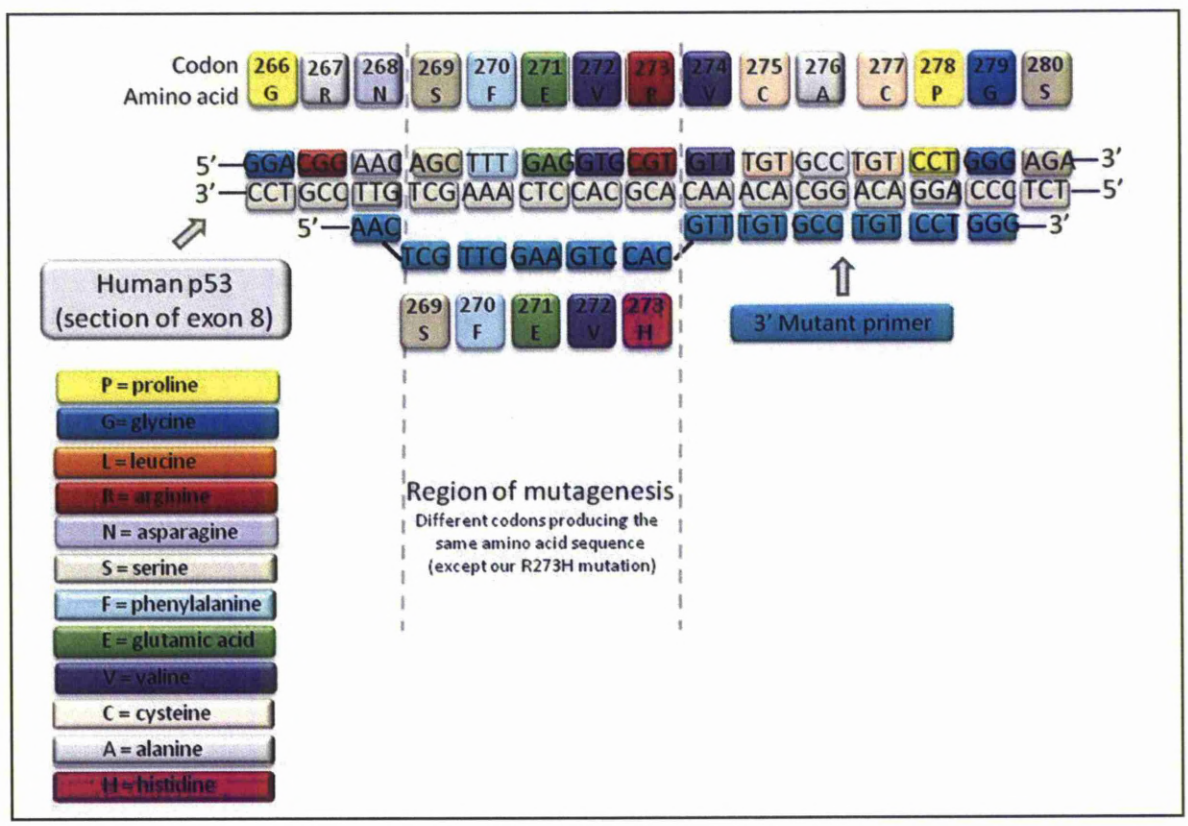



*Figure 0.6: Design of the 3' Mutant for pCEP4p53*

Terminal primers were designed containing KpnI and NotI restriction sites as illustrated in Figures 0.3 to 0.7 to enable sub-cloning into an expression vector: pCEP4.

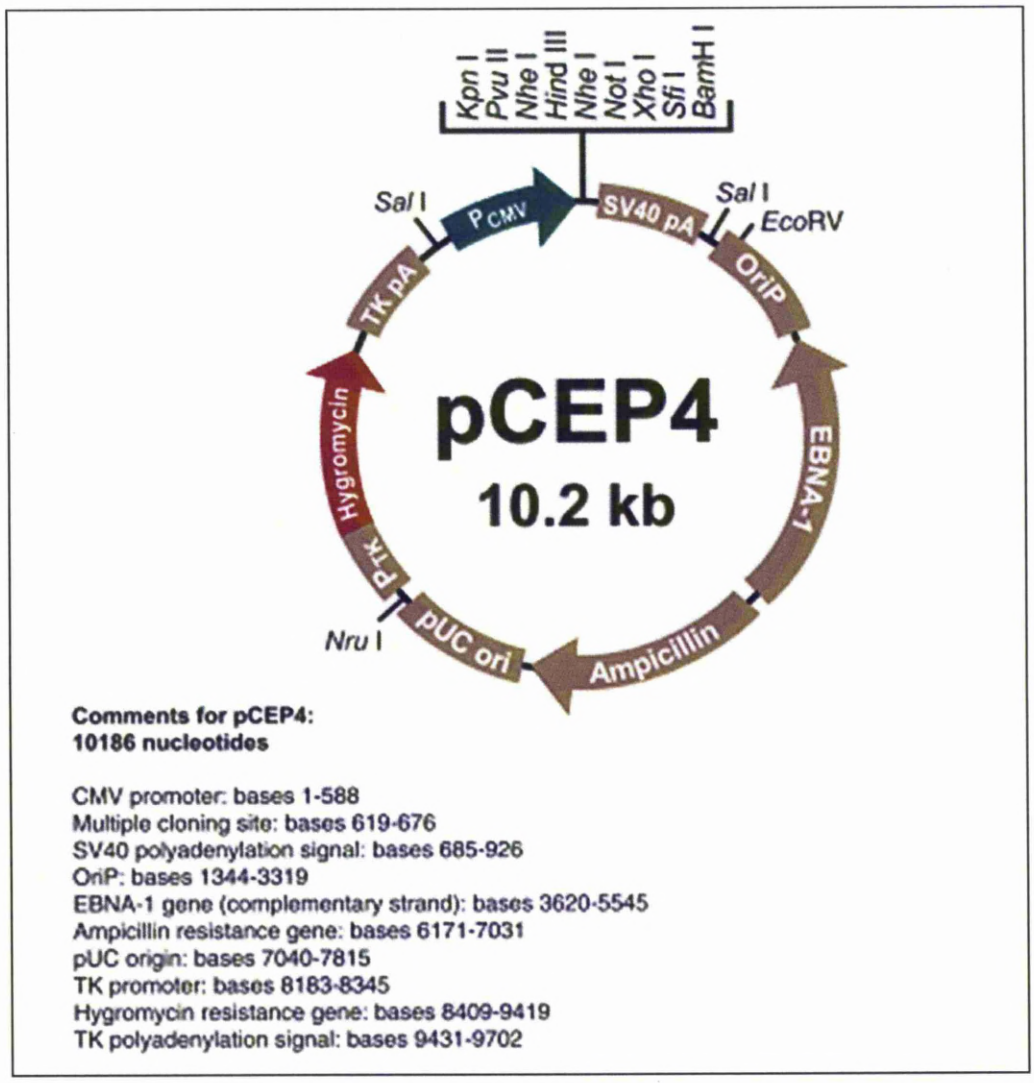



***Figure 0.7: pCEP4 vector.***

***Reproduced from Invitrogen,***
***http://tools.invitrogen.com/content/sfs/manuals/pcep4_man.pdf***

**Optimisation of annealing temperature and cycle number**

Firstly a range of annealing temperatures were tested for thirty cycles of PCR using the 5'end / 3'mutant and the 3'end / 5'mutant primers respectively as shown in Table 0.1:

***Table 0.1: Annealing temperature samples for the 5' end / 3'mutant and the 3' end / 5' mutant primers***

| Sample No. | Primers | DNA | Temperature ($^{o}$C) |
|---|---|---|---|
| **1** | 5'end / 3'M | 100ng pCEPp53 | 53.5 |
| **2** | 5'end / 3'M | 100ng pCEPp53 | 55.7 |
| **3** | 5'end / 3'M | 100ng pCEPp53 | 59.4 |
| **4** | 5'end / 3'M | 100ng pCEPp53 | 63.3 |
| **5** | 5'end / 3'M | Blank | 53.5 |
| **6** | 5'end / 3'M | Blank | 55.7 |
| **7** | 5'end / 3'M | Blank | 59.4 |
| **8** | 5'end / 3'M | Blank | 63.3 |
| **9** | 3'end / 5'M | 100ng pCEPp53 | 53.5 |
| **10** | 3'end / 5'M | 100ng pCEPp53 | 55.7 |
| **11** | 3'end / 5'M | 100ng pCEPp53 | 59.4 |
| **12** | 3'end / 5'M | 100ng pCEPp53 | 63.3 |
| **13** | 3'end / 5'M | Blank | 53.5 |
| **14** | 3'end / 5'M | Blank | 55.7 |
| **15** | 3'end / 5'M | Blank | 59.4 |
| **16** | 3'end / 5'M | Blank | 63.3 |

*Table 0.2: The PCR reaction mix for the 5' end / 3' mutant and the 3' end 5' mutant primer*

| | Samples 1 – 4 (µl) | 5x (µl) A | Samples 5 – 8 (µl) | 5x (µl) B | Samples 9 – 12 (µl) | 5x (µl) C | Samples 13 – 16 (µl) | 5x (µl) D |
|---|---|---|---|---|---|---|---|---|
| 10x AccuPrime Pfx Reaction mix | 5 | 25 | | 25 | 5 | 25 | 5 | 25 |
| Accuprime Pfx DNA polymerase | 1 | 5 | 1 | 5 | 1 | 5 | 1 | 5 |
| 5'end Oligo[0.1µM] | 4.5 | 18 | 4.5 | 18 | | | | |
| 3'end Oligo[0.1µM] | | | | | 4.5 | 18 | 4.5 | 18 |
| 5'Mutant [0.1µM] | | | | | 4.5 | 18 | 4.5 | 18 |
| 3'Mutant [0.1µM] | 4.5 | 18 | 4.5 | 18 | | | | |
| pCEPp53 100ng/µl | 1 | 5 | | | 1 | 5 | | |
| PCR Grade $H_2O$ | 34 | 170 | 35 | 175 | 34 | 170 | 35 | 175 |

Control samples containing all of the PCR components except template DNA were included to detect the presence of DNA contamination. dNTP's were contained within the AccuPrime Pfx reaction mix (Invitrogen) and concentrations of primer and DNA were calculated according to the manufacturer's instructions.

Samples underwent the following regime in a thermal cycler PCR machine (Thermo-Fisher, Px2 thermal cycler)

Stage 1 1 cycle at 95°C for 1 minute (hot start)

Stage 2 Step 1) 94°C for 30 seconds (breaks DNA to single strands)

Step 2) Annealing Temp e.g. 53.5°C (see above)

30 seconds with 15 steps in temperature gradient

Step 3) 68°C for 1 minute (elongation)

This initial PCR reaction was run for thirty cycles and is shown in Figure 0.8.

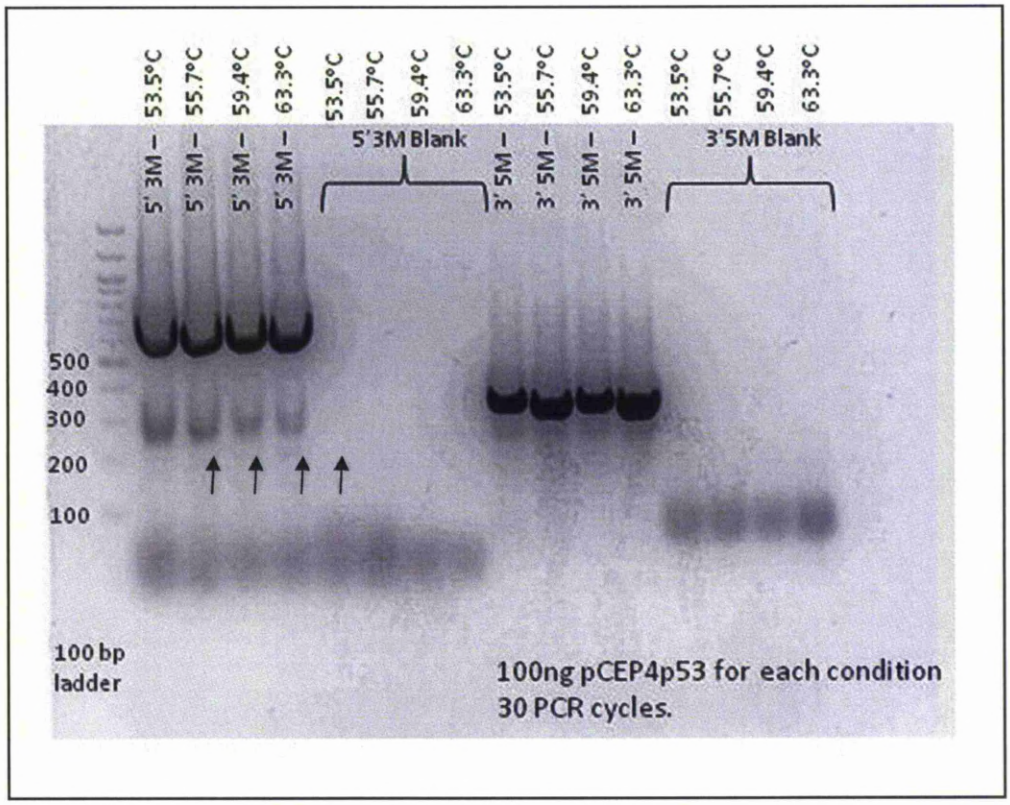



***Figure 0.8: Optimising the annealing temperatures for the 5'end / 3'mutant and the 3'end / 5' mutant primers***

*Figure 0.8 demonstrates the result of PCR amplification at all temperatures for all primers, and with appropriately blank no-template control lanes. 20µl of the PCR products from each of the sixteen samples was combined with 3µl Orange G (2.1.19) and run on a 2% agarose gel, at 80V for 2 hours, against 10µl of 100bp ladder (MBI Fermatas).There were some non-specific bands with the 5' and / 3Mutant samples (indicated by arrows), but these were less pronounced at the higher annealing temperatures.*

The PCR was hence repeated with the same primers and controls at 63.3°C for 5, 10, 15 and 20 cycles. A higher annealing temperature and fewer PCR cycles reduces the error rate and improve the specificity of the primer-binding for the target sequence.

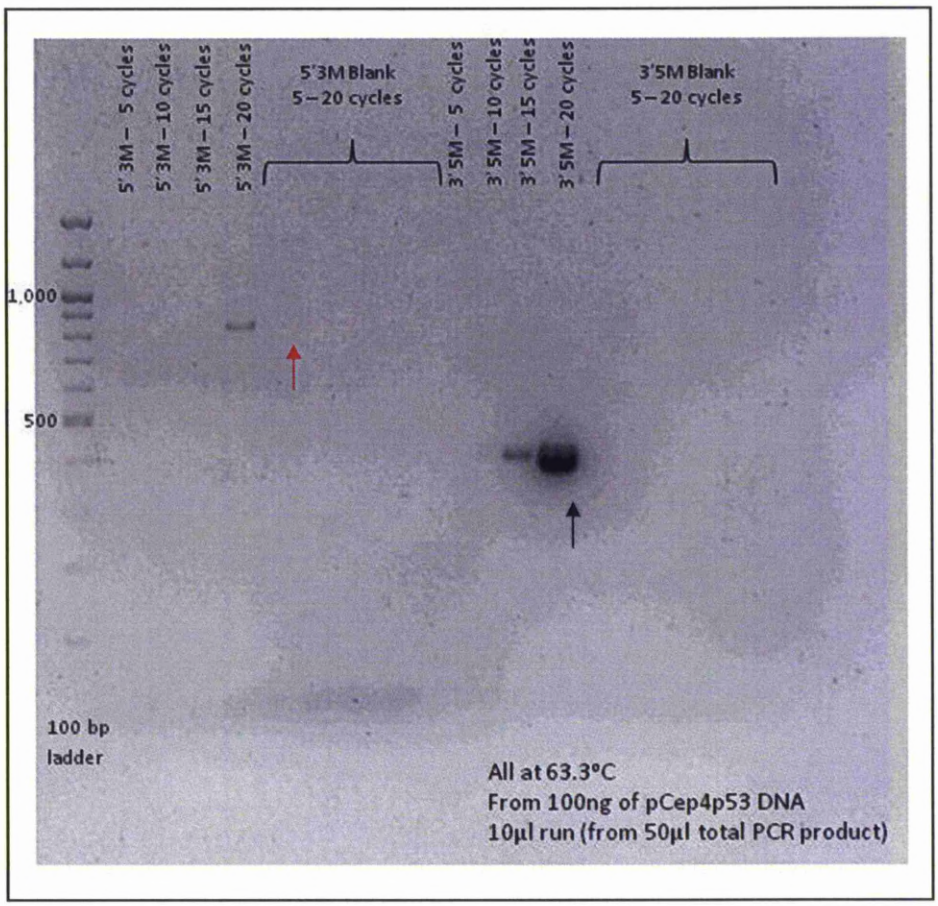



***Figure 0.9: Optimising cycle number using Tann (annealing temperature) =63.3oC for the 5'end / 3'mutant and the 3'end / 5' mutant primers***

*10µl of PCR product per condition was analysed by electrophoresis performed for 1.5 hours at 80V on a 2% agarose gel. Adequate product for subsequent cloning was obtained with 20 cycles of 5'end / 3M only (red arrow), and with 15 cycles of the 3'end /5M sample (black arrow).*

The remaining 40µl of the samples from these two conditions (5'3M - 20 cycles and 3'5M - 15 cycles) was run on a preparative agarose gel and the resulting products were cut from the gel, by visualising the bands under long wave ultraviolet light, to use for the next stage of the mutagenesis process.

Due to time constraints the remainder of the PCR mediated mutagenesis was executed by a colleague following completion of these laboratory studies. The above products underwent GENECLEAN (Q-BIOgene) and were then subjected to further PCR with the 5'end and 3'end primers. After a further GENECLEAN this product was then inserted into the pCEP vector at the KpnI and NotI restriction sites as discussed previously in the Primer Design section.

# References


1. Ferlay, J., Shin, H., Bray, F., Forman, D., Mathers, C., Parkin, D.M., *GLOBOCAN 2008 v1.2, Cancer Incidence and Mortality Worldwide*, in *IARC CancerBase No. 10 [Internet]. Lyon, France. International Agency for Research on Cancer*. 2010.
2. Office for National Statistics, *Cancer Statistics registrations: registrations of cancer diagnosed in 2008, England*. 2008.
3. ISD Online, *Cancer Incidence, Mortality and Survival data*. 2011.
4. Welsh Cancer Intelligence and Surveillance Unit, *Cancer Incidence in Wales*. 2011.
5. Northern Ireland Cancer Registry, *Cancer Incidence and Mortality*. 2011.
6. Office for National Statistics, *Mortality Statistics: Deaths registered in England & Wales*. 2008.
7. General Register Office for Scotland, *Deaths Time Series Data, 1997-2008*. 2010.
8. Northern Ireland Statistics and Research Agency, *Registrar General Annual Report*. 2010.
9. Cancer Research UK - Statistical Information Team, *UK Cancer Information Service. ver 4.3c.001*. 2011.
10. NCIN, *Cancer Incidence by Deprivation England, 1995-2004*. 2008.
11. Marchand, L., *Combined influence of genetic and dietary factors on colorectal cancer incidence in Japanese Americans*. J Natl Cancer Inst Monogr, 1999(26): p. 101-105.
12. Koyama, Y., Kotake, K., *Overview of colorectal cancer in Japan: report from the Registry of the Japanese Society for Cancer of the Colon and Rectum*. Dis Colon Rectum, 1997. **40**(10 Supplement): p. S2-9.
13. Boyle, P., Langman, J. , *ABC of colorectal cancer: Epidemiology*. BMJ, 2000. **321**: p. 805-808.
14. Flood, D., *Colorectal cancer incidence in Asian migrants to the United States and their decendants*. Cancer Causes Control, 2000. **11**(5): p. 403-411.
15. Coleman, M., *Trends in Cancer Incidence and Mortality. IARC Scientific Publications*. 1993, IARC: Lyon.
16. Bray, F., Atkin, W., *International cancer patterns in men: geographical and temporal variations in cancer risk and the role of gender*. JMHG, 2004. **1**(1): p. 38-46.

17. National Cancer Institute, *Surveillance, Epidemiology and End Results (SEER).* 2006.

18. Rachet, B., Maringe, C., Nur, U., Quaresma, M., Shah, A., Woods, L.M., Ellis, L., Walters, S., Forman, D., Steward, J., Coleman, M.P. , *Population-based cancer survival trends in England and Wales up to 2007:an assessment of the NHS cancer plan for England.* The Lancet Oncology 2009.

19. Fuchs, C.S., Giovannucci, E. L., Colditz, G. A., Hunter, D. J., Speizer, F. E., Willett, W. C., *A prospective study of family history and the risk of colorectal cancer.* N Engl J Med, 1994. **331**(25): p. 1669-74.

20. Houlston, R.S., Murday, V., Harocopos, C. , *Screening and genetic counselling for relatives of patients with colorectal cancer in a family cancer clinic.* BMJ, 1990. **301**: p. 366-8.

21. Rowley, P.T., *Screening for an inherited susceptibilty to colorectal cancer.* Genetic testing, 2004. **8**(4): p. 421-430.

22. Wijnen, J.T., Vasen, H. F., Khan, P. M., *Clinical findings with implications for genetic testing in families with clustering of colorectal cancer.* N Engl J Med, 1998. **339**: p. 511-18.

23. Vasen, H., Moslein, G, ALonso, A., *Guidelines for the clinical management of familial adenomatous polyposis (FAP).* . Gut, 2008. **57**: p. 704-13.

24. Vasen, H., van, BM, Buskens, E., *A cost-effectiveness analysis of colorectal screening of hereditary nonpolyposis colorectal carcinoma gene carriers.* Cancer Cell, 1998. **82**: p. 1632-7.

25. Cairns, S.R., Scholefield, J.H., Steele, R.J., *Guidelines for colorectal cancer screening and surveillance in moderate and high risk groups (update from 2002).* Gut, 2010. **59**: p. 666-690.

26. Dunlop, M.G., *Guidance on large bowel surveillance for people with two first degree relatives with colorectal cancer or one first degree relative diagnosed with colorectal cancer under 45 years.* 2002. p. 17v-20.

27. Burkitt, D.P., *Epidemiology of cancer of the colon and rectum. 1971.* Dis Colon Rectum, 1993. **36**(11): p. 1071-82.

28. Rooney, P.S., Hunt, L., Clarke, P.A., *Wheat fibre, lactulose and rectal mucosal proliferation in individuals with a family history of colorectal cancer.* 1984. **Br J Surg**(81): p. 1792-4.

29. Modan, B., Barell, V., Lubin, F., *Low fibre intake as an aetiological factor in cancer of the colon.* J Natl Cancer Inst Mono, 1975(55): p. 15-18.

30. Peters, U., Sinha, R., Chattergee, N., *Dietary fibre and colorectal adenoma in a colorectal cancer detection programme.* Lancet, 2003. **361**(1491-95).

31. Norat, T., Bingham, S., Ferrari, P., *Meat, fish and colorectal cancer risk: the European Prosepective Investigation into cancer and nutrition.* J Natl Cancer Inst., 2005. **97**: p. 906-916.

32. Imray, C.H., Radley, S., Davis, A., Barker, G., Hendrickse, C. W., Donovan, I. A., Lawson, A. M., Baker, P. R., Neoptolemos, J. P., *Faecal unconjugated bile acids in patients with colorectal cancer or polyps.* Gut, 1992. **33**(9): p. 1239-45.

33. Schottenfeld, D., Winawer, S.J., *Cholecystectomy and colorectal cancer.* Gastroenterology, 1983. **85**(4): p. 966-7.

34. Zhao, C., Ge, Z., Wang, Y., *Meta-analysis of observational studies on cholecystectomy and the risk of colorectal adenoma.* European Journal of Gastroenterology & Hepatology, 2012. **24**(4): p. 375-81.

35. Weingarten, M.A., Zalmanovici, A., Yaphe, J., *Dietary calcium supplementation for preventing colorectal cancer and adenomatous polyps.* Cochrane Database Syst Rev, 2005(3): p. CD003548.

36. Bjelke E, *Epidemiologic studies of cancer of the stomach, colon and rectum with special emphasis on the role of diets*, in *Thesis Vols I-IV*. 1973, University of Minnesota.

37. Thornalley, P., *More good things in vegetables than you ever imagined.* Biochemist 1999(21): p. 19-23.

38. Thornalley, P.J., *Isothiocyanates: mechanism of cancer chemopreventive action.* Anticancer Drugs, 2002. **13**(4): p. 331-8.

39. Franeschi, S., Dal-Maso, L., Augustin, L., *Dietary glycaemic load and colorectal cancer risk.* Ann Oncol 2001(12): p. 173-8.

40. Gonzalez, C.A., *Nutrition and cancer: the current epidemiological evidence.* Br J Nutr, 2006(Aug 96 Suppl 1): p. S42-5.

41. IARC Working group on the Evaluation of Cancer Preventative agents, *Weight control and physical activity*. 2002, Lyon: International agency for research on cancer.

42. Giovannucci, E., Colditz, G.A., Stampfer, M.J., Willet, W.C., *Physical activity, obesity and risk of colorectal cancer in women (United States).* Cancer Causes Control, 1996(7): p. 253-63.

43. Cho, E., Smith-Warner, SA, Ritz, J., *Alcohol intake and colorectal cancer: a pooled analysis of 8 cohort studies.* Ann Intern Med., 2004(140): p. 603-613.

44. Cole BF, L., RF, Halabi S, et al., *Aspirin for the chemprevention of colorectal adenomas: meta-analysis of the randomized trials.* J Natl Cancer Inst Monogr, 2009(101): p. 256-266.

45. Baron, J.A., Sandler, R.S., Bresalier, R.S., *A randomised trial of rofecoxib for the chemoprevention of colorectal adenomas.* Gastroenterology, 2006(131): p. 1674-1682.

46. Chlebowski, R.T., Wactawski-Wende, J., Ritenbaugh, C., Hubbell, F. A., Ascensao, J., Rodabough, R. J., Rosenberg, C. A., Taylor, V. M., Harris, R., Chen, C., Adams-Campbell, L. L., White, E., *Estrogen plus progestin and colorectal cancer in postmenopausal women.* N Engl J Med, 2004. **350**(10): p. 991-1004.

47. Solimando, R., Bazzoli, F., Ricciardiello, L., *Chemoprevention of colorectal cancer: A role for ursodeoxycholic acid, folate and hormone replacement treatment.* Best Pract Res Clin Gastroenterol., 2011. **25**(4-5): p. 555-68.

48. Bach, S.P., Renehan, A.G., Potten, C.S., *Stem cells: the intestinal stem cell as a paradigm.* Carcinogenesis, 2000(21): p. 469-476.

49. Luebeck, E.G., Moolgavker, S.H.,, *Multistage carinogenesis and the incidence of colorectal cancer.* Proc. Natl. Acad. Sci. USA, 2002(99): p. 15095-15100.

50. Boland, C.R., Sato, J., Appleman, H.D., Bresalier, R.S. and Feinberg, A.P., *Microallelotyping defines thr sequences and tempo of allelic losses at tumour supressor gene loci during colorectal cancer progression.* Nat. Med., 1995(1): p. 902-909.

51. O'Brien, M.J., *Colorectal polyps.* Cancer of the Colon, Rectum and Anus. 1995, New York: McGraw Hill inc. 127-135.

52. Rembacken, B.J., Fujii, T., Cairns, A., *Flat and depressed colonic neoplasms: a prospective study of 1000 colonoscopies in the UK.* Lancet, 2000(355): p. 1211-4.

53. Chandler, J., Lagasse, E., *Cancerous stem cells: deviant stem cells with cancer-causing misbehavior.* Stem Cell Research & Therapy 2010. **1**: p. 13.

54. Dukes, C., *The classification of cancer of the rectum.* Journal of Pathological Bacteriology, 1932. **35**: p. 323.

55. Dukes, C.E., Bussey, H.J.R., *The spread of rectal cancer and its effect on prognosis.* Br J Cancer, 1958(12): p. 309-20.

56. Astler, V.B., Coller, F.A., *The prognostic significance of direct extension of carcinoma of the colon and rectum.* Annals of Surgery, 1954. **139**: p. 846.

57. Edge, S.B., Byrd, D.R., Compton, C.C. (Eds). *AJCC (American Joint Committee on Cancer) Cancer Staging Manual, 7th ed, Springer, New York.* 2010, AJCC (American Joint Committee on Cancer). p. 143.

58. Cedermark, B.J., Shultz, S.S., Bakshi, S., *The value of liver scan in the follow-up study of patients with adenocarcinoma of the colon and rectum.* Surg Gynecol Obstet, 1977(144): p. 745-8.

59. Mella, J., Biffin, A., Radcliffe, A. G., Stamatakis, J. D., Steele, R. J., *Population-based audit of colorectal cancer management in two UK health regions. Colorectal Cancer Working Group, Royal College of Surgeons of England Clinical Epidemiology and Audit Unit.* Br J Surg, 1997. **84**(12): p. 1731-6.

60. Hewitson P, G.P., Towler B, *Screening for colorectal cancer using the faecal occult blood test, hemocult.* The Cochrane Database of Systematic Reviews., 2007.

61. The UK CRC Screening Pilot Evaluation Team, *Evaluation of the UK Colorectal Cancer Screening Pilot Final Report* 2003.

62. The UK CRC Screening Pilot Evaluation Team, *English Pilot of Bowel Cancer Screening: an evaluation of the second round. Final Report to the Department of Health* 2006.

63. Atkin, W.S., Edwards, R., Kralj-Hans, I., *Once-only flexible sigmoidoscopy screening in prevention of colorectal cancer: a multicentre randomised controlled trial.* . The Lancet, 2010. **375**(9726): p. 1624-1633.

64. Thompson, M.R., Heath, I., Ellis, B.G., Swarbrick, E.T., Wood, L.F., Atkin, W.S., *Identifying and managing patients at low risk of bowel cancer in general practice.* BMJ, 2003(327): p. 263-5.

65. ACPGBI, *Guidelines for the Manangement of Colorectal Cancer (3rd edition).* 2007.

66. Enker, W.E., *Extent of operations for large bowel cancer*, in *Large Bowel Cancer. Clinical surgery international*, J.J. DeCosse, Editor. 1981, Churchill Livingstone: Edinburgh. p. 78-93.

67. Kapiteijn, E., Marijnen, C.A., Nagtegaal, I.D., Putter, H., Steup, W., Wigger, T. , *Preoperative radiotherapy combined with total mesorectal excision for respectable rectal cancer.* N Engl J Med, 2001. **345**: p. 638-646.

68. Sebag Montefiore, D., Steele, R., Quirke, P., *Routine short course pre-op radiotherapy or selected post–op chemoradiotherapy for resectable rectal cancer/ Preliminary results of the MRC CR07 randomised trial.* Journal of Clinical Oncology, Ann Soc Clin Oncol, 2006. **Part 1**(24): p. 18S.

69. Rutman, R.J., Cantarow, A., Paschkis, K.E., *Studies on 2-acetylaminoflourene carcinogenesis:III. The utililization of uracil-2-$C^{14}$ by pre-neoplastic rat liver.* Cancer Res., 1954. **14**: p. 119.

70. Wohlhueter, R.M., McIvor, R.S., Plagemann, P.G., *Facilitated transport of uracil and 5-fluorouracil, and permeation of orotic acid into cultured mammalian cells.* J. Cell Physiol., 1980. **104**: p. 309–319.

71. Diasio, R.B., Harris, B.E., *Clinical pharmacology of 5-fluorouracil.* Clin. Pharmacokinet., 1989. **16**: p. 215–237.

72. Johnston, P.G., Kaye, S. , *Capecitabine: a novel agent for the treatment of solid tumors.* . Anticancer Drugs 2001. **12**: p. 639–646.

73. Schuller, J., *Preferential activation of capecitabine in tumor following oral administration to colorectal cancer patients.* Cancer Chemother. Pharmacol., 2000. **45**: p. 291–297.

74. Wiseman, L.R., Adkins, J.C., Plosker, G.L., *Oxaliplatin. A review of its use in the management of metastatic colorectal cancer.* Drugs and Aging, 1999. **14**: p. 459-75.

75. Saris, C.P., VanDeVart, P. J., Rietbroek, R. C., Blommanert, F. A., *In vitro formation of DNA adducts by cisplatin, lobaplatin and oxaliplatin in calf thymus DNA in solution and in cultured human cells.* Carcinogenesis, 1996. **17**(12): p. 2763-2769.

76. Scheeff, E.D., Briggs, J. M., Howell, S. B., *Molecular modeling of the intrastrand guanine-guanine DNA adducts produced by cisplatin and oxaliplatin.* Mol. Pharmacol, 1999. **56**(3): p. 633-643.

77. Raymond, E., Faivre, S., Woynarowski, J.M., Chaney, S.G., *Oxaliplatin: mechanism of action and antineoplastic activity.* Semin. Oncol, 1998. **25**(2 Suppl. 5): p. 4-12.

78. Allen, J., Graham, M.A., Firth, J., *Biotransformation and pharmacokinetic analysis of oxaliplatin in patients with advanced gastrointestinal cancer.* Proc. Am. Assoc. Cancer Res, 1998. **39**: p. 159

79. Haydon, A., *Adjuvant chemotherapy in colon cancer: what is the evidence?* Intern Med J, 2003. **33**(3): p. 119-24.

80. NICE, *Improving outcomes in colorectal cancers: manual update. Guidance on cancer services.* 2004.

81. Lembersky, B.C., Wieand, H.S., Petrelli, N.J., *Oral uracil and tegafur plus leucovorin compared with intravenous fluorouracil and leucovorin in stage II and III carcinoma of the colon: results from National Surgical Adjuvant Breast and Bowel Project Protocol C-06.* J Clin Oncol., 2006. **24**(13): p. 2059-64.

82. Twelves, C., Wong, A., Nowacki, M.P., *Capecitabine as adjuvant treatment for stage III colon cancer.* N Engl J Med 2005. **352**: p. 2696-2704.

83. Wolmark, N., *Provocative optimism in the treatment of early stage disease.* Oncologist, 2005. **10 Suppl 2**: p. 1-3.

84. Andre, T., deGramont, A., *An overview of adjuvant systemic chemotherapy for colon cancer.* Clin Colorectal Cancer, 2004. **4 Suppl 1**: p. S22-8.

85. International Multicentre Pooled Analysis of B2 Colon Cancer Trials (IMPACT B2) Investigators, *Efficacy of adjuvant fluorouracil and folinic acid in B2 colon cancer.*

*International Multicentre Pooled Analysis of B2 Colon Cancer Trials (IMPACT B2) Investigators.* J Clin Oncol, 1999. **17**(5): p. 1356-63.

86. Wolmark, N., et al., *Clinical trial to assess the relative efficacy of fluorouracil and leucovorin, fluorouracil and levamisole, and fluorouracil, leucovorin, and levamisole in patients with Dukes' B and C carcinoma of the colon: results from National Surgical Adjuvant Breast and Bowel Project C-04.* J Clin Oncol, 1999. **17**(11): p. 3553-9.
87. Simmonds, P.C., *Palliative chemotherapy for advanced colorectal cancer: systematic review and meta-analysis. Colorectal Cancer Collaborative Group.* BMJ, 2000. **321**(7260): p. 531-5.
88. Grothey, A., Kleeberg, U. R., Stauch, M., Hieke, K., *Health economic analysis of fluoropyrimidine-based therapies of colorectal cancer from the perspective of statutory sickness funds.* Z Gastroenterol, 2005. **43**(2): p. 155-61.
89. Hurwitz, H., et al., *Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer.* N Engl J Med, 2004. **350**(23): p. 2335-42.
90. Cunningham, D., Humblet, Y., Siena, S., Khayat, D., Bleiberg, H., Santoro, A., Bets, D., Mueser, M., Harstrick, A., Verslype, C., Chau, I., Van Cutsem, E., *Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer.* N Engl J Med, 2004. **351**(4): p. 337-45.
91. Muto, T., Bussey, H.J., Morson, B.C., *The evolution of cancer of the colon and rectum.* Cancer, 1975. **36**(6): p. 2251-2270.
92. Fearon, E.R., Vogelstein B., *A genetic model for colorectal tumorigenesis.* Cell, 1990. **61**(5): p. 759-767.
93. Smith, G., Carey, F. A., Beattie, J., Wilkie, M. J., Lightfoot, T. J., Coxhead, J., Garner, R. C., Steele, R. J., Wolf, C. R., *Mutations in APC, Kirsten-ras, and p53--alternative genetic pathways to colorectal cancer.* Proc Natl Acad Sci U S A, 2002. **99**(14): p. 9433-8.
94. Narayan, S., Roy, D., *Role of APC and DNA mismatch repair genes in the development of colorectal cancers.* Mol Cancer, 2003. **2**: p. 41.
95. Bodmer, W.F., Bailey, C. J., Bodmer, J., Bussey, H. J., Ellis, A., Gorman, P., Lucibello, F. C., Murday, V. A., Rider, S. H., Scambler, P., *Localization of the gene for familial adenomatous polyposis on chromosome 5.* Nature, 1987. **328**(6131): p. 614-6.
96. Solomon, E., Voss, R., Hall, V., Bodmer, W. F., Jass, J. R., Jeffreys, A. J., Lucibello, F. C., Patel, I., Rider, S. H., *Chromosome 5 allele loss in human colorectal carcinomas.* Nature, 1987. **328**(6131): p. 616-9.

97. Cottrell, S., Bicknell, D., Kaklamanis, L., Bodmer, W. F., *Molecular analysis of APC mutations in familial adenomatous polyposis and sporadic colon carcinomas.* Lancet, 1992. **340**(8820): p. 626-30.

98. Nakamura, Y., *The role of the adenomatous polyposis coli (APC) gene in human cancers.* Adv Cancer Res, 1993. **62**: p. 65-87.

99. Powell, S.M., Zilz, N., Beazer-Barclay, Y., Bryan, T. M., Hamilton, S. R., Thibodeau, S. N., Vogelstein, B., Kinzler, K. W., *APC mutations occur early during colorectal tumorigenesis.* Nature, 1992. **359**(6392): p. 235-7.

100. Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., *Identification and characterization of the familial adenomatous polyposis coli gene.* Cell, 1991. **66**(3): p. 589-600.

101. Spirio, L., Olschwang, S., Groden, J., Robertson, M., Samowitz, W., Joslyn, G., Gelbert, L., Thliveris, A., Carlson, M., Otterud, B., *Alleles of the APC gene: an attenuated form of familial polyposis.* Cell, 1993. **75**(5): p. 951-7.

102. Lynch, H.T., de la Chapelle, A., *Hereditary colorectal cancer.* . N. Engl. J. Med. , 2003. **348**: p. 919-32.

103. Rustgi, A.K., *The genetics of hereditary colon cancer.* Genes Dev., 2007. **21**: p. 2525-38.

104. Morin, P.J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., Kinzler, K. W., *Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC.* Science, 1997. **275**(5307): p. 1787-90.

105. Rubinfeld, B., Albert, I., Porfiri, E., Munemitsu, S., Polakis, P., *Loss of beta-catenin regulation by the APC tumor suppressor protein correlates with loss of structure due to common somatic mutations of the gene.* Cancer Res, 1997. **57**(20): p. 4624-30.

106. Fukata, M., Abreu, M. T., *Microflora in colorectal cancer: a friend to fear.* Nature Medicine, 2010. **16**: p. 639-641.

107. Bos, J.L., *ras oncogenes in human cancer: a review.* Cancer Res, 1989. **49**(17): p. 4682-9.

108. Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M., Bos, J. L., *Genetic alterations during colorectal-tumor development.* N Engl J Med, 1988. **319**(9): p. 525-32.

109. Van Cutsem, E., et al., *Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status.* J Clin Oncol. **29**(15): p. 2011-9.

110. Andreyev, H.J., et al., *Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study.* Br J Cancer, 2001. **85**(5): p. 692-6.

111. Andreyev, H.J., et al., *Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study.* J Natl Cancer Inst, 1998. **90**(9): p. 675-84.

112. Wood, L.D., Parsons, D.W., Jones, S., Lin, J., Sjoblom, T., *The genomic landscapes of human breast and colorectal cancers.* Science, 2007. **318**: p. 1108–13.

113. Shibata, D., Reale, M. A., Lavin, P., Silverman, M., Fearon, E. R., Steele, G., Jessup, J. M., Loda, M., Summerhayes, I. C., *The DCC protein and prognosis in colorectal cancer.* N Engl J Med, 1996. **335**(23): p. 1727-32.

114. Saito, M., Yamaguchi, A., Goi, T., Tsuchiyama, T., Nakagawara, G., Urano, T., Shiku, H., Furukawa, K., *Expression of DCC protein in colorectal tumors and its relationship to tumor progression and metastasis.* Oncology, 1999. **56**(2): p. 134-41.

115. Vasen, H.F., Mecklin, J. P., Khan, P. M., Lynch, H. T., *The International Collaborative Group on HNPCC.* Anticancer Res, 1994. **14**(4B): p. 1661-4.

116. Vasen, H.F., Watson, P., Mecklin, J. P., Lynch, H. T., *New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC.* Gastroenterology, 1999. **116**(6): p. 1453-6.

117. Herman, J.G., Umar, A., Polyak, K., Graff, J. R., Ahuja, N., Issa, J. P., Markowitz, S., Willson, J. K., Hamilton, S. R., Kinzler, K. W., Kane, M. F., Kolodner, R. D., Vogelstein, B., Kunkel, T. A., Baylin, S. B., *Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma.* Proc Natl Acad Sci U S A, 1998. **95**(12): p. 6870-5.

118. Soreide, K., Janssen, E. A., Soiland, H., Korner, H., Baak, J. P., *Microsatellite instability in colorectal cancer.* Br J Surg, 2006. **93**(4): p. 395-406.

119. Popat, S., Hubner, R., Houlston, R. S., *Systematic review of microsatellite instability and colorectal cancer prognosis.* J Clin Oncol, 2005. **23**(3): p. 609-18.

120. Haddad, R., Ogilvie, R. T., Croitoru, M., Muniz, V., Gryfe, R., Pollet, A., Shanmugathasan, P., Fitzgerald, T., Law, C. H., Hanna, S. S., Jothy, S., Redston, M., Gallinger, S., Smith, A. J., *Microsatellite instability as a prognostic factor in resected colorectal cancer liver metastases.* Ann Surg Oncol, 2004. **11**(11): p. 977-82.

121. Kim, W.H., Suh, J. H., Kim, T. I., Shin, S. K., Paik, Y. H., Chung, H. W., Kim, D. Y., Jeong, J. H., Kang, J. K., Kim, H., Kim, N. K., *Colorectal flat neoplasia.* Dig Liver Dis, 2003. **35**(3): p. 165-71.

122. Adachi, M., Muto, T., Okinaga, K., Morioka, Y., *Clinicopathologic features of the flat adenoma.* Dis Colon Rectum, 1991. **34**(11): p. 981-6.

123. Lanspa, S.J., Rouse, J., Smyrk, T., Watson, P., Jenkins, J. X., Lynch, H. T., *Epidemiologic characteristics of the flat adenoma of Muto. A prospective study.* Dis Colon Rectum, 1992. **35**(6): p. 543-6.

124. Sakashita, M., Aoyama, N., Maekawa, S., Kuroda, K., Shirasaka, D., Ichihara, T., Kuroda, Y., Minami, R., Maeda, S., Kasuga, M., *Flat-elevated and depressed, subtypes of flat early colorectal cancers, should be distinguished by their pathological features.* Int J Colorectal Dis, 2000. **15**(5-6): p. 275-81.

125. Fujimori, T., Satonaka, K., Yamamura-Idei, Y., Nagasako, K., Maeda, S., *Non-involvement of ras mutations in flat colorectal adenomas and carcinomas.* Int J Cancer, 1994. **57**(1): p. 51-5.

126. Ruggero, D., *The role of Myc-induced protein synthesis in cancer.* Cancer Res, 2009. **69**: p. 8839–43.

127. Pelengaris, S., Khan, M., Evan, G., *c-MYC: More than just a matter of life and death.* Nature Cancer Reviews, 2002. **2**: p. 764-776.

128. Fearon, E.R., *Molecular genetics of colorectal cancer.* Annu Rev Pathol. **6**: p. 479-507.

129. Vogelstein, B., Lane, D., Levine, A. J., *Surfing the p53 network.* Nature, 2000. **408**(6810): p. 307-10.

130. Lane, D.P., Crawford, L. V., *T antigen is bound to a host protein in SV40-transformed cells.* Nature, 1979. **278**(5701): p. 261-3.

131. Linzer, D.I., Levine, A. J., *Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells.* Cell, 1979. **17**(1): p. 43-52.

132. DeLeo, A.B., Jay, G., Appella, E., Dubois, G. C., Law, L. W., Old, L. J., *Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse.* Proc Natl Acad Sci U S A, 1979. **76**(5): p. 2420-4.

133. Kress, M., May, E., Cassingena, R., May, P., *Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum.* J Virol, 1979. **31**(2): p. 472-83.

134. Maltzman, W., Czyzyk, L., *UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells.* Mol Cell Biol, 1984. **4**(9): p. 1689-94.

135. Rotter, V., Wolf, D., Nicolson, G. L., *The expression of transformation-related protein p53 and p53-containing mRNA in murine RAW117 large cell lymphoma cells of differing metastatic potential.* Clin Exp Metastasis, 1984. **2**(3): p. 199-204.

136. Benchimol, S., Lamb, P., Crawford, L. V., Sheer, D., Shows, T. B., Bruns, G. A., Peacock, J., *Transformation associated p53 protein is encoded by a gene on human chromosome 17.* Somat Cell Mol Genet, 1985. **11**(5): p. 505-10.

137. Finlay, C.A., Hinds, P.W., Levine, A.J. , *The p53 proto-oncogene can act as a suppressor of transformation.* Cell 1989. **57**: p. 1083–1093.

138. Lane, D.P., *Cancer.p53, guardian of the genome.* Nature, 1992. **358**: p. 15-16.

139. Donehower, L.A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., Bradley, A., *Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours.* Nature, 1992. **356**(6366): p. 215-21.

140. El-Deiry, W.S., Tokino, T., Velculascu, V.E., *WAF1, a potential mediator of p53 tumor suppression.* Cell 1993. **75**(4): p. 817-25.

141. Haupt, Y., Maya, R., Kazaz, A. , *MdM2 promotes the rapid degradation of p53.* Nature, 1997. **387**: p. 296-299.

142. Kubbutat, M.H., Jones, S.N,, Vousden, K.H., *Regulation of p53 stability by Mdm2.* Nature, 1997. **387**: p. 299-303.

143. Anzola, M., Burgos, J., *p53 protein structure.* Expert Reviews in Molecular Medicine, 2003. **5**.

144. Hainaut, P., Hollstein, M., *p53 and human cancer: the first ten thosand mutations.* Adv. Cancer Res, 2000. **77**: p. 81-137.

145. Brooks, C.L., Gu, W., *Ubiquitination, phophorylation,and acetylation: the molecular basis for p53 regulation.* Curr. Opin. Cell Biol., 2003. **15**: p. 164-171.

146. Bieging, K.T., Attardi, L.D., *Deconstructing p53 transcriptional networks in tumor suppression.* Trends in Cell Biology, 2011. **22**(2): p. 97-106.

147. Levine AJ, *p53, the Cellular Gatekeeper for Growth and Division.* Cell 2000. **88**(3): p. 323-331.

148. Kruse, J.P., Gu, W., *Snapshot: p53 posttranslational modifications.* Cell 2008. **133**: p. 930.

149. Appella, E., Anderson, C.W., *Post-translational modifications and activation of p53 by genotoxic stresses.* Eur. J. Biochem., 2001. **268**: p. 2764-2772.

150. Shieh, S.Y., Ikeda, M., Taya, Y., *DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2.* Cell, 1997. **91**: p. 325-334.

151. Michael, D., Oren, M., *The p53-Mdm2 module and the ubiquitin system.* Semin. Cancer Biol., 2003. **13**: p. 49-58.

152. Honda. R, Tanaka, H., Yasuda, H., *Oncoprotein MDM2 is a ubiquitin ligase E3 for tumour suppressor p53.* FEBS lett., 1997. **420**: p. 25-27.

153. Ringshausen, I., Shea, C.C., Finch A.J. , *Mdm2 is critically and continously required to suppress lethal p53 activity in vivo.* Cancer cell, 2006. **10**: p. 501-514.

154. Dornan, D., Wertz, I., Shimizu, H. , *The ubiquitin ligase COP1 is a critical negative regulator of p53.* Nature, 2004. **429**: p. 86-92.

155. Leng, R.P., Lin, Y., Ma, W., *Pirh2, a p53 induced ubiquitin-protein ligase, promoted p53 degradation.*. Cell Growth Differ, 2003. **112**: p. 779-791.

156. Chen, D., Kon, N., Li, M., *ARF-BP1/Mule is a critical mediator of the ARf tumour suppressor.* Cell Growth Differ, 2005. **121**: p. 1071-1083.

157. Lowe, S.W., Sherr, C.J., *Tumour suppression by Ink4a-Arf: progress and puzzles.* Curr. Opin. Genet. Dev., 2003. **13**: p. 77-83.

158. Sherr CJ, *Divorcing Arf and p53: an unsettled case.* Nat. Rev. Cancer., 2006. **6**: p. 663-673.

159. Marine, J.C., Jochemsen, A.G., *Mdmx as an essential regulator of p53 activity.* . Biochem. Biophys. Res. Commun., 2005. **331**(750-760).

160. Kurz, E.U., Lees-Miller, S.P., *DNA damage induced activation of ATM and ATM dependent signaling pathways.* . DNA Repair (Amst), 2004. **3**: p. 889-900.

161. Goodman, R.H., Smolik, S., *CBP/p300 in cell growth, transformation and development.* Genes Dev., 2000. **14**: p. 1553-1577.

162. Taylor, W.R., Stark, G.R., *Regulation of the G2/M transition by p53.* Oncogene, 2001. **20**(15): p. 1803-15.

163. Yin, C., *Bax suppresses tumorigenesis and stimulates apoptosis in vivo.* Nature, 1997. **385**: p. 637-640.

164. Oda, E., Ohki, R., Murasawa, H. Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Takana, N. , *Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis.* Science, 2000. **288**: p. 1053-1058.

165. Nakano, K., Vousden, K., *PUMA, a novel proaptotic gene, is induced by p53.* Mol. Cell, 2001. **7**: p. 683-694.

166. Polyak, K., Xia, Y., Zweier, J.L., *A model for p53-induced apoptosis.* Nature, 1997. **389**: p. 300-305.

167. Wu, G.S., Kim, K., el-Deiry, W.S., *KILLER/DR5, a novel DNA-damage inducible death receptor gene, links the p53-tumor suppressor to caspase activation and apoptotic death.* Adv. Exp. Med. Biol., 2000. **465**: p. 143-51.

168. Lin, Y., Ma, W., Benchimol, S. , *Pidd, a new death-domain-containing protein, is induced by p53 and promotes apoptosis.* . NAt. Genet., 2000. **26**: p. 122-127.

169. Smith, M.L., Chen, I.T., Zhan, Q., *Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen.* Science, 1994. **266**(5189): p. 1376-80.

170. Tanaka, H., Arakawa, H., Yamaguchi, T., *A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage.* Nature, 2000. **404**: p. 42-49.

171. Monpezat, J.P., Delattre, O., Bernard, A., Grunwald, D., Remvikos, Y., Muleris, M., Salmon, R. J., Frelat, G., Dutrillaux, B., Thomas, G., *Loss of alleles on chromosome*

*18 and on the short arm of chromosome 17 in polyploid colorectal carcinomas.* Int J Cancer, 1988. **41**(3): p. 404-8.

172. Baker, S.J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., vanTuinen, P., Ledbetter, D. H., Barker, D. F., Nakamura, Y., White, R., Vogelstein, B., *Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas.* Science, 1989. **244**(4901): p. 217-21.

173. Rodrigues, N.R., Rowan, A., Smith, M. E., Kerr, I. B., Bodmer, W. F., Gannon, J. V., Lane, D. P., *p53 mutations in colorectal cancer.* Proc Natl Acad Sci U S A, 1990. **87**(19): p. 7555-9.

174. Baker, S.J., Markowitz, S., Fearon, E. R., Willson, J. K., Vogelstein, B., *Suppression of human colorectal carcinoma cell growth by wild-type p53.* Science, 1990. **249**(4971): p. 912-5.

175. Purdie, C.A., O'Grady, J., Piris, J., Wyllie, A. H., Bird, C. C., *p53 expression in colorectal tumors.* Am J Pathol, 1991. **138**(4): p. 807-13.

176. Soong, R., Powell, B., Elsaleh, H., Gnanasampanthan, G., Smith, D. R., Goh, H. S., Joseph, D.,Llacopetta, B., *Prognostic significance of TP53 gene mutation in 995 cases of colorectal carcinoma. Influence of tumour site, stage, adjuvant chemotherapy and type of mutation.* Eur J Cancer, 2000. **36**(16): p. 2053-60.

177. Borresen-Dale, A.L., Lothe, R. A., Meling, G. I., Hainaut, P., Rognum, T. O., Skovlund, E., *TP53 and long-term prognosis in colorectal cancer: mutations in the L3 zinc-binding domain predict poor survival.* Clin Cancer Res, 1998. **4**(1): p. 203-10.

178. Dix, B., Robbins, P., Carrello, S., House, A., Iacopetta, B., *Comparison of p53 gene mutation and protein overexpression in colorectal carcinomas.* Br J Cancer, 1994. **70**(4): p. 585-90.

179. Kikuchi-Yanoshita, R., Konishi, M., Ito, S., Seki, M., Tanaka, K., Maeda, Y., Iino, H., Fukayama, M., Koike, M., Mori, T., *Genetic changes of both p53 alleles associated with the conversion from colorectal adenoma to early carcinoma in familial adenomatous polyposis and non-familial adenomatous polyposis patients.* Cancer Res, 1992. **52**(14): p. 3965-71.

180. Kressner, U., Inganas, M., Byding, S., Blikstad, I., Pahlman, L., Glimelius, B., Lindmark, G., *Prognostic value of p53 genetic changes in colorectal cancer.* J Clin Oncol, 1999. **17**(2): p. 593-9.

181. Beroud, C., Soussi, T., *The UMD-p53 database: new mutations and analysis tools.* Hum Mutat, 2003. **21**(3): p. 176-81.

182. Soussi, T., Dehouche, K., Beroud, C., *p53 website and analysis of p53 gene mutations in human cancer: forging a link between epidemiology and carcinogenesis.* Hum Mutat, 2000. **15**(1): p. 105-13.

183. Bazan, V., Migliavacca, M., Tubiolo, C., Macaluso, M., Zanna, I., Corsale, S., Amato, A., Calo, V., Dardanoni, G., Morello, V., La Farina, M., Albanese, I., Tomasino, R. M., Gebbia, N., Russo, A., *Have p53 gene mutations and protein expression a different biological significance in colorectal cancer?* J Cell Physiol, 2002. **191**(2): p. 237-46.

184. Goh, H.S., Yao, J., Smith, D. R., *p53 point mutation and survival in colorectal cancer patients.* Cancer Res, 1995. **55**(22): p. 5217-21.

185. Bosari, S., Marchetti, A., Buttitta, F., Graziani, D., Borsani, G., Loda, M., Bevilacqua, G., Coggi, G., *Detection of p53 mutations by single-strand conformation polymorphisms (SSCP) gel electrophoresis. A comparative study of radioactive and nonradioactive silver-stained SSCP analysis.* Diagn Mol Pathol, 1995. **4**(4): p. 249-55.

186. Leahy, D.T., Salman, R., Mulcahy, H., Sheahan, K., O'Donoghue, D. P., Parfrey, N. A., *Prognostic significance of p53 abnormalities in colorectal carcinoma detected by PCR-SSCP and immunohistochemical analysis.* J Pathol, 1996. **180**(4): p. 364-70.

187. Manne, U., Weiss, H. L., Myers, R. B., Danner, O. K., Moron, C., Srivastava, S., Grizzle, W. E., *Nuclear accumulation of p53 in colorectal adenocarcinoma: prognostic importance differs with race and location of the tumor.* Cancer, 1998. **83**(12): p. 2456-67.

188. Soong, R., Grieu, F., Robbins, P., Dix, B., Chen, D., Parsons, R., House, A., Iacopetta, B., *p53 alterations are associated with improved prognosis in distal colonic carcinomas.* Clin Cancer Res, 1997. **3**(8): p. 1405-11.

189. Adrover, E., Maestro, M. L., Sanz-Casla, M. T., del Barco, V., Cerdan, J., Fernandez, C., Balibrea, J. L., *Expression of high p53 levels in colorectal cancer: a favourable prognostic factor.* Br J Cancer, 1999. **81**(1): p. 122-6.

190. Ahnen, D.J., Feigl, P., Quan, G., Fenoglio-Preiser, C., Lovato, L. C., Bunn, P. A., Stemmerman, G., Wells, J. D., Macdonald, J. S., Meyskens, F. L., *Ki-ras mutation and p53 overexpression predict the clinical behavior of colorectal cancer: a Southwest Oncology Group study.* Cancer Res, 1998. **58**(6): p. 1149-58.

191. Munro, A.J., Lain, S., Lane, D. P., *P53 abnormalities and outcomes in colorectal cancer: a systematic review.* Br J Cancer, 2005. **92**(3): p. 434-44.

192. Russo, A., Bazan, V., Lacopetta, B., Kerr, D., Soussi, T., Gebbia, N., *The TP53 colorectal cancer international collaborative study on the prognostic and predictive*

*significance of p53 mutation: influence of tumor site, type of mutation, and adjuvant treatment.* J Clin Oncol, 2005. **23**(30): p. 7518-28.

193. Bunz, F., Hwang, P.M., Torrance, C. , *Disruption of p53 in human cancer cells alters the responses to therapeutic agents.* J Clin Invest, 1999. **104**: p. 263-269.

194. Breivik, J., Lothe, R.A., Meling, G.I., *Different genetic pathways to proximal and distal colorectal cancer influenced by sex-related factors.* Int J Cancer, 1997. **74**(664-669).

195. Elsaleh, H., Joseph, D., Grieu, F., *Association of tumour site and sex with survival benefit from adjuvant chemotherapy in colorectal cancer.* Lancet, 2000. **355**: p. 1745-1750.

196. Boyer, J., McLean, E.G., Aroori, S., *Characterization of p53 wild-type and null isogenic colorectal cancer cell lines resistant to 5-fluorouracil, oxaliplatin, and irinotecan.* Clin Cancer Res 2004. **10**(6): p. 2158-2167.

197. Toscano, F., Parmentier, B., Fajoui, Z.E., *53 dependent and independent sensitivity to oxaliplatin of colon cancer cells.* . Biochem Pharmacol, 2007. **74**(3): p. 392-406.

198. Zaanan, A., Cuilliere-Dartigues, P., Guilloux, A. , *Impact of p53 expression and microsatellite instability on stage III colon cancer disease-free survival in patients treated by 5-fluorouracil and leucovorin with or without oxaliplatin.* Ann Oncol, 2010. **21**(4): p. 772-80.

199. Chen, Y., Chen, P. L., Lee, W. H., *Hot-spot p53 mutants interact specifically with two cellular proteins during progression of the cell cycle.* Mol Cell Biol, 1994. **14**(10): p. 6764-72.

200. Shaulsky, G., Goldfinger, N., Rotter, V., *Alterations in tumor development in vivo mediated by expression of wild type or mutant p53 proteins.* Cancer Res, 1991. **51**(19): p. 5232-7.

201. Wang, X.J., Greenhalgh, D. A., Jiang, A., He, D., Zhong, L., Brinkley, B. R., Roop, D. R., *Expression of a p53 mutant in the epidermis of transgenic mice accelerates chemical carcinogenesis.* Oncogene, 1998. **17**(1): p. 35-45.

202. Hsiao, M., Low, J., Dorn, E., Ku, D., Pattengale, P., Yeargin, J., Haas, M., *Gain-of-function mutations of the p53 gene induce lymphohematopoietic metastatic potential and tissue invasiveness.* Am J Pathol, 1994. **145**(3): p. 702-14.

203. Hussain, S.P., Harris, C. C., *Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes.* Cancer Res, 1998. **58**(18): p. 4023-37.

204. Sigal, A., Rotter, V., *Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome.* Cancer Res, 2000. **60**(24): p. 6788-93.

205. Scian, M.J., Stagliano, K. E., Ellis,, et al., *Tumor-derived p53 mutants induce oncogenesis by transactivating growth-promoting genes.* Oncogene, 2004. **23**(25): p. 4430-43.

206. Prives, C., Hall, P. A., *The p53 pathway.* J Pathol, 1999. **187**(1): p. 112-26.

207. Chene, P., *In vitro analysis of the dominant negative effect of p53 mutants.* J Mol Biol, 1998. **281**(2): p. 205-9.

208. de Vries, A., Flores, E. R., Miranda, B., Hsieh, H. M., van Oostrom, C. T., Sage, J., Jacks, T., *Targeted point mutations of p53 lead to dominant-negative inhibition of wild-type p53 function.* Proc Natl Acad Sci U S A, 2002. **99**(5): p. 2948-53.

209. Harvey, M., Vogel, H., Morris, D., Bradley, A., Bernstein, A., Donehower, L. A., *A mutant p53 transgene accelerates tumour development in heterozygous but not nullizygous p53-deficient mice.* Nat Genet, 1995. **9**(3): p. 305-11.

210. Lavigueur, A., Maltby, V., Mock, D., Rossant, J., Pawson, T., Bernstein, A., *High incidence of lung, bone, and lymphoid tumors in transgenic mice overexpressing mutant alleles of the p53 oncogene.* Mol Cell Biol, 1989. **9**(9): p. 3982-91.

211. Mantovani, R., *A survey of 178 NF-Y binding CCAAT boxes.* Nucleic Acids Res, 1998. **26**(5): p. 1135-43.

212. Frazier, M.W., He, X., Wang, J., Gu, Z., Cleveland, J. L., Zambetti, G. P., *Activation of c-myc gene expression by tumor-derived p53 mutants requires a discrete C-terminal domain.* Mol Cell Biol, 1998. **18**(7): p. 3735-43.

213. Cadwell, C., Zambetti, G. P., *The effects of wild-type p53 tumor suppressor activity and mutant p53 gain-of-function on cell growth.* Gene, 2001. **277**(1-2): p. 15-30.

214. Olive, K.P., Tuveson, D. A., Ruhe, Z. C., Yin, B., Willis, N. A., Bronson, R. T., Crowley, D., Jacks, T., *Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome.* Cell, 2004. **119**(6): p. 847-60.

215. Lang, G.A., Iwakuma, T., Suh, Y. A., Liu, G., Rao, V. A., Parant, J. M., Valentin-Vega, Y. A., Terzian, T., Caldwell, L. C., Strong, L. C., El-Naggar, A. K., Lozano, G., *Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome.* Cell, 2004. **119**(6): p. 861-72.

216. Benard, J., Douc-Rasy, S., Ahomadegbe, J. C., *TP53 family members and human cancers.* Hum Mutat, 2003. **21**(3): p. 182-91.

217. Soussi, T., *Focus on the p53 gene and cancer: advances in TP53 mutation research.* Hum Mutat, 2003. **21**(3): p. 173-5.

218. Flores, E.R., Sengupta, S., Miller, J. B., Newman, J. J., Bronson, R., Crowley, D., Yang, A., McKeon, F., Jacks, T., *Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family.* Cancer Cell, 2005. 7(4): p. 363-73.

219. Li, Y., Prives, C., *Are interactions with p63 and p73 involved in mutant p53 gain of oncogenic function?* Oncogene, 2007. **26**: p. 2220-2225.

220. Lang, G.A., *Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome.* Cell, 2004. **119**: p. 861-872.

221. Bergamaschi, D., *p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis.* Cancer Cell, 2003. **3**: p. 387-402.

222. Di Agostino, S., *The disruption of the protein complex mutantp53/p73 increases selectively the response of tumor cells to anticancer drugs.* Cell Cycle, 2008. **7**: p. 3440-3447.

223. Kravchenko, J.E., *Small-molecule RETRA suppresses mutant p53-bearing cancer cells through a p73-dependent salvage pathway.* Proc. Natl Acad. Sci. USA, 2008. **105**: p. 6302-6307.

224. Brosh, R., Rotter, V., *When mutants gain new powers: news from the mutant p53 field.* Nat Rev Cancer, 2009. **9**(10): p. 701-13.

225. Terzian, T., *The inherent instability of mutant p53 is alleviated by Mdm2 or p16INK4a loss.* Genes Dev., 2008. **22**: p. 1337-1344.

226. Gualberto, A., Aldape, K., Kozakiewicz, K., Tlsty, T. D., *An oncogenic form of p53 confers a dominant, gain-of-function phenotype that disrupts spindle checkpoint control.* Proceedings of the National Academy of Sciences, 1998. **95**(9): p. 5166-5171.

227. Iwamoto, K.S., Mizuno, T., Ito, T., Tsuyama, N., Kyoizumi, S., Seyama, T., *Gain-of-function p53 mutations enhance alteration of the T-cell receptor following X-irradiation, independently of the cell cycle and cell survival.* Cancer Res, 1996. **56**(17): p. 3862-5.

228. Murphy, K.L., Dennis, A. P., Rosen, J. M., *A gain of function p53 mutant promotes both genomic instability and cell survival in a novel p53-null mammary epithelial cell model.* Faseb J, 2000. **14**(14): p. 2291-302.

229. El-Hizawi, S., Lagowski, J. P., Kulesz-Martin, M., Albor, A., *Induction of gene amplification as a gain-of-function phenotype of mutant p53 proteins.* Cancer Res, 2002. **62**(11): p. 3264-70.

230. Caulin, C., Nguyen, T., Lang, G. A., Goepfert, T. M., Brinkley, B. R., Cai, W. W., Lozano, G., Roop, D. R., *An inducible mouse model for skin cancer reveals distinct roles for gain- and loss-of-function p53 mutations.* J Clin Invest, 2007. **117**(7): p. 1893-901.

231. Lotem, J., Sachs, L., *A mutant p53 antagonizes the deregulated c-myc-mediated enhancement of apoptosis and decrease in leukemogenicity.* Proc Natl Acad Sci U S A, 1995. **92**(21): p. 9672-6.

232. Peled, A., Zipori, D., Rotter, V., *Cooperation between p53-dependent and p53-independent apoptotic pathways in myeloid cells.* Cancer Res, 1996. **56**(9): p. 2148-56.

233. Fisher, D.E., *Apoptosis in Cancer Therapy: Crossing the Threshold.* Cell, 1994(78): p. 539-542.

234. Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., Craig, R. W., *Participation of p53 protein in the cellular response to DNA damage.* Cancer Res, 1991. **51**(23 Pt 1): p. 6304-11.

235. Blandino, G., Levine, A. J., Oren, M., *Mutant p53 gain of function: differential effects of different p53 mutants on resistance of cultured cells to chemotherapy.* Oncogene, 1999. **18**(2): p. 477-85.

236. Li, Y., Guessous, F., Kwon, S., Kumar, M., Ibidapo, O., Fuller, L., Johnson, E., Lal, B., Hussaini, I., Bao, Y., Laterra, J., Schiff, D., Abounader, R., *PTEN has tumor-promoting properties in the setting of gain-of-function p53 mutations.* Cancer Res, 2008. **68**(6): p. 1723-31.

237. Napoli, C., Lemieux, C., Jorgensen, R., *Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans.* Plant Cell, 1990. **2**(4): p. 279-289.

238. Romano, N., Macino, G., *Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences.* Mol Microbiol, 1992. **6**(22): p. 3343-53.

239. Guo, S., Kemphues, K. J., *par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed.* Cell, 1995. **81**(4): p. 611-20.

240. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., Mello, C. C., *Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans.* Nature, 1998. **391**(6669): p. 806-11.

241. Meister, G., Tuschl, T., *Mechanisms of gene silencing by double-stranded RNA.* Nature, 2004. **431**(7006): p. 343-9.

242. Lewis, B.P., Burge, C. B., Bartel, D. P., *Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets.* Cell, 2005. **120**(1): p. 15-20.

243. Calin, G.A., Sevignani, C., Dumitru, C. D., Hyslop, T., Noch, E., Yendamuri, S., Shimizu, M., Rattan, S., Bullrich, F., Negrini, M., Croce, C. M., *Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 2999-3004.

244. Martinez, L.A., Naguibneva, I., Lehrmann, H., Vervisch, A., Tchenio, T., Lozano, G., Harel-Bellan, A., *Synthetic small inhibiting RNAs: efficient tools to inactivate oncogenic mutations and restore p53 pathways.* Proc Natl Acad Sci U S A, 2002. **99**(23): p. 14849-54.

245. Vikhanskaya, F., Lee, M. K., Mazzoletti, M., Broggini, M., Sabapathy, K., *Cancer-derived p53 mutants suppress p53-target gene expression--potential mechanism for gain of function of mutant p53.* Nucleic Acids Res, 2007. **35**(6): p. 2093-104.

246. Mulkeen, A.L., Silva, T., Yoo, P. S., Schmitz, J. C., Uchio, E., Chu, E., Cha, C., *Short interfering RNA-mediated gene silencing of vascular endothelial growth factor: effects on cellular proliferation in colon cancer cells.* Arch Surg, 2006. **141**(4): p. 367-74; discussion 374.

247. Klampfer, L., Swaby, L. A., Huang, J., Sasazuki, T., Shirasawa, S., Augenlicht, L., *Oncogenic Ras increases sensitivity of colon cancer cells to 5-FU-induced apoptosis.* Oncogene, 2005. **24**(24): p. 3932-41.

248. Brummelkamp, T.R., Bernards, R., Agami, R., *A system for stable expression of short interfering RNAs in mammalian cells.* Science, 2002. **296**(5567): p. 550-3.

249. Bossi, G., Lapi, E., Strano, S., Rinaldo, C., Blandino, G., Sacchi, A., *Mutant p53 gain of function: reduction of tumor malignancy of human cancer cell lines through abrogation of mutant p53 expression.* Oncogene, 2006. **25**(2): p. 304-9.

250. Behlke, M.A., *Progress towards in vivo use of siRNAs.* Mol Ther, 2006. **13**(4): p. 644-70.

251. Kim, B., Tang, Q., Biswas, P. S., Xu, J., Schiffelers, R. M., Xie, F. Y., Ansari, A. M., Scaria, P. V., Woodle, M. C., Lu, P., Rouse, B. T., *Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor pathway genes: therapeutic strategy for herpetic stromal keratitis.* Am J Pathol, 2004. **165**(6): p. 2177-85.

252. Shen, J., Samul, R., Silva, R. L., Akiyama, H., Liu, H., Saishin, Y., Hackett, S. F., Zinnen, S., Kossen, K., Fosnaugh, K., Vargeese, C., Gomez, A., Bouhana, K., Aitchison, R., Pavco, P., Campochiaro, P. A., *Suppression of ocular neovascularization with siRNA targeting VEGF receptor 1.* Gene Ther, 2006. **13**(3): p. 225-34.

253. Harlow, E., Lane, D., *Using antibodies : a laboratory manual.* 1999, Cold Spring Harbour Laboratory Press: Cold Spring Harbour

254. Brummelkamp, T.R., Bernards, R., Agami, R., *Stable suppression of tumorigenicity by virus-mediated RNA interference.* Cancer Cell, 2002. **2**(3): p. 243-7.